Maternal Serum Markers, Placental Pathophysiology and Down's Syndrome Pregnancies

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Abstract

Down's syndrome (DS) is the most common congenital cause of mental retardation and is caused by trisomy of chromosome 21. Initially prenatal diagnosis of chromosome abnormalities was offered only to pregnant women aged 35 years or over. In the last decade, maternal serum screening programmes have been introduced across the UK to give a risk of a pregnancy being affected of DS. These screening programmes are largely based on measurement of maternal serum concentrations of alpha-fetoprotein, human chorionic gonadotrophin (hCG) and also, in some cases, unconjugated estriol and inhibin-A, combined with maternal age. Despite being routinely used to screen for DS pregnancies, little is understood about the mechanisms that underlie the alterations in maternal serum levels of these markers. It was the aim of this study to examine maternal serum markers of DS synthesised by the placenta. This mainly focused on the elevation in hCG and inhibin-A in DS pregnancies.

Placental levels and localisation of inhibin-A and activin-A were studied in control and DS pregnancies, as well as the maternal serum concentrations of both markers. Levels of inhibin-A and activin-A were significantly elevated in DS placental extracts (1.46MoM and 1.62MoM respectively) and staining intensity of the inhibin/activin β_A and α subunits tended to be stronger in DS placentae. This suggested that elevated maternal serum levels of both markers in DS had their origin in increased placental synthesis, similar to previous observations on elevated placental production of hCG in DS. Dimeric inhibin-A has recently been introduced as a screening marker of DS in some programmes and the elevation in maternal serum inhibin-A to 2.06MoM reported in the current study is similar to previous reports. The use of activin-A as a screening marker for DS was investigated by other groups during the course of the current study and it was noted that the small degree of elevation and the overlap in levels between control and DS makes it of little use as a screening marker. This was independently confirmed by the current study which noted an elevation in maternal serum level of activin-A in DS pregnancies to 1.26MoM.

Growth factors known to have opposing effects on hCG secretion from placental trophoblast cells (epidermal growth factor (EGF) and transforming growth factor β_1 (TGFβ_1)) were studied in maternal serum, placentae, maternal urine and amniotic fluid from control and DS pregnancies. It was discovered that amniotic fluid levels of EGF

and TGFβ_1 were significantly reduced in DS pregnancies to 0.5MoM and 0.685MoM respectively. Maternal urine levels of EGF were significantly reduced (0.726MoM), as were placental levels of TGFβ_1 (0.675MoM) in DS pregnancies. It was postulated that reduced TGFβ_1 levels in DS placentae could have a bearing on placental hCG secretion since TGFβ_1 has an inhibitory effect on hCG secretion from and differentiation of cytotrophoblast cells. Reduced TGFβ_1 could result in a diminished inhibitory effect of this growth factor on hCG secretion. A reduction in amniotic fluid EGF and TGFβ_1 was an interesting finding, particularly in the case of EGF. EGF is not produced by the placenta suggesting that amniotic fluid EGF is likely to be of fetal origin, with reduced levels of EGF indicating abnormal fetal synthesis of this factor. Reduced urinary EGF but normal maternal serum levels of EGF was a surprising finding since maternal urine is a filtrate of maternal blood. It was postulated that this could be due to rapid maternal clearance of EGF secreted by the fetus resulting in the reduction in EGF secretion by the fetus being detectable only in amniotic fluid and maternal urine. This is the first study to analyse EGF and TGFβ_1 in control and DS pregnancies and the results suggest that fetal and placental growth may be affected by altered levels of these growth factors. However, it was also recognised that altered levels of EGF and TGFβ_1 may be a result of abnormal placental or fetal function in DS.

To further study placental function in normal and DS pregnancies, a widely used method of isolating and culturing placental trophoblasts was optimised using term placentae. Further immunopurification of the isolated cells by immunodepletion was also carried out and the percentage purity of the cytotrophoblasts before and after immunopurification was confirmed using a panel of antibodies. The differentiation of cytotrophoblasts was monitored by immunocytochemistry and hCG and placental alkaline phosphatase (PLAP) secretion into the culture medium. The effect of cryopreservation on hCG and PLAP secretion by trophoblast cultures was also studied and it was found that hCG secretion from the cultures was significantly inhibited ($p=0.025$) and the viability of the cells was reduced by cryopreservation although PLAP secretion was unaltered ($p>0.05$). It was the aim of this study to use the isolated cells to study hCG secretion by differentiating cytotrophoblasts under different culture conditions.

Because of the limited availability of trophoblast cells, different methods of determining hCG mRNA concentrations were compared: northern blot analysis, Real Time PCR and

a custom developed ELISA based assay. It was found that northern blot analysis was impractical because too much RNA was required for each analysis and it was extremely time consuming to carry out. The ELISA based method that was developed was a specific and quick method of mRNA quantification and results from this assay correlated with those obtained using Real Time PCR. The most efficient method of mRNA quantification was Real Time PCR because it required the least amount of starting RNA and was quick to carry out. Availability of the equipment to carry out this method of mRNA quantification could, however, prove difficult.

The genes for interferon (IFN) α and β receptors are localised to chromosome 21. It was postulated that over-expression of these receptors could contribute to altered placental function in DS, as there is evidence that IFNα administration increases hCG secretion from hCG secreting bladder carcinoma cell lines. The effect of IFNα on hCG secretion from normal placentae is poorly understood, consequently the current study investigated the effect of IFNα on hCG secretion by normal term placental trophoblast cultures. It was discovered that IFNα administration to trophoblasts failed to alter hCG secretion ($p>0.05$) or hCG mRNA production ($p>0.05$). The situation in mid trimester placentae could however be different because the rate of hCG secretion at this stage is higher than at term and also the largest difference between control and DS maternal serum hCG levels is found at this stage of gestation.

In conclusion, this study has illustrated that the DS placenta and fetus do not function normally in DS and that maternal serum levels of the markers studied appear to reflect altered placental secretion of these factors. Further studies that focus on chromosome 21 markers (e.g. IFN receptors, superoxide dismutase or stress 70 protein chaperone) may help to further understand the link between the extra copy of chromosome 21 and the alterations seen in maternal serum markers in DS pregnancies.

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Declaration

Excerpts from the results of this thesis have been published as detailed on page 22. I certify that this thesis does not contain any other material published or written by another person except where due reference is made in the text. The results presented in this thesis have not been submitted for any other degree or diploma.

Gillian Dalgliesh

Excerpts and preliminary results of this thesis have been published and presented as detailed below:

Publications

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Abbreviations

+ve	positive
-ve	negative
4-PL	four parameter logistic
ACTH	adrenocorticotophic hormone
ActR	activin receptor
ADP	adenosine diphosphate
AF	amniotic fluid
AFP	alpha-fetoprotein
Amps	amperes
APS	ammonium persulphate
ATP	adenosine triphosphate
α2M	alpha 2 macroglobulin
BSA	bovine serum albumin
b	base
bp	base pair
°C	degrees Celsius
cDNA	coding DNA
cm	centimetre
conc	concentrated
Ct	threshold cycle
CVS	chorionic villus sampling
DAB	diaminodenzidine
dCTP	deoxycytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
DIG	digoxygenin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleoside triphosphate
DPBS	Dulbecco's phosphate buffered saline
DS	Down's syndrome
DTT	dithiothreitol

EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
ELISA	enzyme linked immunosorbent assay
EVT	extravillous cytotrophoblast
FAM	carboxy-fluorescein
fβ-hCG	free beta hCG
FBS	fetal bovine serum
FISH	fluorescence in situ hybridisation
FITC	fluorescein isothiocyanate
FSH	follicle stimulating hormone
g	gram
g	gravitational force
GAPDH	glyceraldehyde-6-phosphate dehydrogenase
GGT	gamma glutamyl transferase
GH	growth hormone
GnRH	gonadotrophin releasing hormone
HBSS	Hank's balanced salt solution
hCG	human chorionic gonadotrophin
HCl	hydrochloric acid
H-hCG	hyperglycosylated hCG
hPL	human placental lactogen
HRP	horse radish peroxidase
HSP 70	heatshock protein 70
IFNGR2	interferon gamma receptor 2
IFN-R1	type 1 interferon receptor
IFN-R2	type 2 interferon receptor
IFNα	interferon alpha
IFNβ	interferon beta
IFNγ	interferon gamma
ihCG	intact hCG
ISRE	interferon stimulated response element
IUGR	intrauterine growth retardation
Kb	kilobase
kDa	kilodalton

l	litre
LDL	low density lipoprotein
LH	leytenising hormone
log	logarithm
M	molar (moles per litre)
M199	medium 199
MESA	MOPS-EDTA sodium acetate
mg	milligram
min	minute
mIU	milli international unit
ml	millilitre
mM	millimolar (millimoles per litre)
MoM	multiples of the median
MOPS	3-(N-Morpholino) propanesulphonic acid
mRNA	messenger RNA
MS	maternal serum
mU	milliunits
MU	maternal urine
μg	microgram
μl	microlitre
μm	micrometer
μM	micromolar (micromoles per litre)
n	number
N	normal
NCS	newborn calf serum
ng	nanogram
NGS	normal goat serum
nm	nanometres
nM	nanomolar (nanomoles per litre)
p	significance value
PAPP-A	pregnancy associated plasma protein A
PBS	phosphate buffered saline
PBST	phosphate buffered saline & tween
PCR	polymerase chain reaction
pg	picogram

PLAP	placental alkaline phosphatase
PMSF	phenylmethylsulfonyl fluoride
PPIA	cyclophilin A
Pro MBP	pro eosinophil major basic protein
PTGFB	placental transforming growth factor beta
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
RT-PCR	reverse transcriptase PCR
rpm	revolutions per minute
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
sec	second
SOD	superoxide dismutase
SP-1	schwangerschafts protein 1
SSC	sodium citrate sodium chloride
STCH	stress 70 protein chaperone
STET	sucrose triton-x-100 EDTA tris
T13	trisomy 13
T18	trisomy 18
T21	trisomy 21
TAE	tris acetate EDTA
TAMRA	tetramethylrhodamine
TBST	tris buffered saline & tween
TE	tris EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TET	tetrachloro-fluorescein
TGF	transforming growth factor
TM	transmembrane
TMB	tetramethylbenzidine
TOP	termination of pregnancy
TSH	thyrotrophin stimulating hormone
U	units
UE_3	unconjugated estriol
UV	ultraviolet

"None but those who have experienced them can conceive of the enticements of science. In other studies you go as far as others have gone before you, and there is nothing more to know; but in a scientific pursuit there is a continual food for discovery and wonder."

Dr Victor Frankenstein

In Frankenstein *or* The Modern Prometheus,

Mary Shelly (1831).

Section 1

Introduction

1.1 Down's Syndrome

1.1.1 Cytogenetic Basis of Down's Syndrome

Down's Syndrome (DS) is the most common congenital cause of severe mental retardation. Without the intervention of prenatal screening and diagnosis, between 1 in 600 and one in 700 births would be affected with DS (Crawfurd, 1988). Most cases are the result of trisomy 21, with the extra chromosome usually arising due to non-disjunction. In two thirds of cases, non-disjunction occurs during the first meiotic phase in females with the remainder of cases split equally between the second phase of meiosis in the female and both meiotic phases in the male. In 5% of cases, the extra genetic material arises due to a translocation, when part of chromosome 21 is attached to another chromosome. This is usually a Robertsonian translocation that can only occur between acrocentric chromosomes. It is thought that a specific region of the long arm of chromosome 21 (band 21q22), often called the "Down's syndrome critical region", is sufficient to produce the DS phenotype when it is present in extra dosage.

1.1.2 Down's Syndrome Phenotype

The phenotype of an individual affected with DS is expressed both physically and mentally. These individuals have severe mental handicap, with an IQ of around fifty. They tend to have a shorter stature and a shorter, broader face with flat features. Their eyes are slanted with white spots on the iris and they have a characteristic simian palm. Weak muscles often make physical development of DS children slower than normal and around forty to fifty percent have congenital heart defects. They have an increased susceptibility to respiratory infections due to small air passages, and an impaired immune system which can also lead to persistent ear infections. They are also prone to developing acute lymphatic leukaemia, early onset Alzheimer's type dementia and type 1 diabetes, all of which have been associated with genes on chromosome 21 (Burch and Milunsky, 1969). Those with DS tend to have a shorter than average life span, although some do live to beyond middle age when hypothyroidism and dementia become a problem (Scoggin and Patterson, 1982). Most are sexually underdeveloped and sterile however some women with DS have children fifty percent of whom would also be expected to have DS.

1.1.3 Incidence of Down's Syndrome

The incidence of DS, like other chromosomal abnormalities such as trisomy 18, trisomy 13 and Kleinfelter's syndrome (47XXY), increases with the mother's advancing age. The risk increases from 1 in 1,528 at the age of twenty to 1 in 384 at thirty five, and 1 in 6 at fifty years of age (Cuckle et al. 1987). Over the last decade, more and more couples have been waiting until their mid thirties before having a child. The proportion of women at an increased risk of having a DS has therefore increased and many of these women opt for prenatal diagnosis. The fact that there is no way to prevent or cure this disorder also means that following prenatal diagnosis of DS, avoidance of the birth of an affected child is dependant upon termination of pregnancy.

1.2 Prenatal Diagnosis

1.2.1 Amniocentesis

The most common way of detecting chromosomally abnormal pregnancies is to perform an amniocentesis. This is usually carried out at around fifteen to sixteen weeks of pregnancy and involves removing 10 to 20 ml of amniotic fluid (AF) via a needle inserted through the abdominal wall into the amniotic sac. The fluid contains enzymes, skin cells and excretions from the fetus. The fetal cells (amniocytes) are cultured to allow fetal chromosome analysis. Alternatively, a quicker method of detecting chromosomal abnormality is to use fluorescence *in situ* hybridisation (FISH) on uncultured amniocytes. The presence of extra or missing chromosomes is detected using probes, specific for certain areas of a chromosome, coupled to a fluorochrome (Morris et al. 1999).

1.2.2 Chorionic Villous Sampling (CVS)

To allow fetal karyotyping in the first trimester of pregnancy, a different method called chorionic villous sampling (CVS) may be employed. This involves the removal of some chorion frondosum, either transcervically via a cannula or endoscope, or transabdominally as with amniocentesis. Fetal chromosome analysis is carried out using direct analysis of metaphases or following culturing of villi. The results of this test are available much earlier than those from amniocentesis, at around ten to fourteen weeks of gestation compared to around sixteen weeks of gestation. This early

diagnosis means that a termination is much safer to carry out using aspiration methods rather than induction of labour, should the chromosome analysis show an abnormality.

1.2.3 Drawbacks of Diagnostic Tests

There are risks to the developing fetus associated with performing both tests. There is an additional miscarriage risk of approximately 1 percent associated with performing an amniocentesis (Smidt-Jensen et al. 1992). It has been shown that a transabdominal CVS carries a risk of miscarriage of around 2 percent, while a transcervical CVS carries a risk of around 7 percent (Smidt-Jensen et al. 1992). These risks combined with the financial cost of carrying out many tests means that it is desirable to select mothers who are believed to be at high risk of having an abnormal pregnancy before offering fetal chromosome analysis. Initially, selection was based solely on the mother's age but this only detected around 30 percent of DS pregnancies (Wald et al. 1988b). Now the result of biochemical tests carried out on the mother's blood, combined with maternal age, is used to select those at high risk of carrying a chromosomally abnormal fetus. If a high risk is reported following these screening tests, then an invasive diagnostic procedure is offered to either confirm or rule out fetal abnormality.

1.3 Prenatal Screening

As part of routine prenatal care, all expectant mothers in Scotland are offered a serum screening test for neural tube defects and DS in the second trimester of pregnancy. Because only a small sample of maternal blood is required to perform these tests, there is no risk to the pregnancy. Prenatal screening tests have been developed over a number of years with input from different groups working in this field. (Aitken and Crossley, 1992). These tests involve measuring maternal serum concentrations of factors secreted into the maternal circulation following synthesis by either the fetus or placenta. These results are converted to an odds ratio that the pregnancy is affected and combined with the maternal age risk (Spencer, 1995; Zimmermann et al. 1996). In the west of Scotland the biochemical screening programme, which started in 1991, measures the maternal serum concentrations of alpha-fetoprotein (AFP) and intact human chorionic gonadotrophin (ihCG). If a risk equal to or greater than 1:220 is determined, an amniocentesis is offered. This test detects 68% of DS pregnancies, with a false positive

rate of 5.2% (Aitken and Crossley, 1997), compared with only 30-35% of DS pregnancies being detected by offering an amniocentesis based solely on a maternal age of greater than thirty-five years (Aitken and Crossley, 1992). In some other regions of the UK these markers are combined with unconjugated estriol (UE_3) and/or inhibin-A. More recently, first trimester screening for chromosomal abnormalities has been introduced. The principal maternal serum markers of DS pregnancies in the first trimester are pregnancy associated plasma protein A (PAPP-A) and the free beta subunit of hCG (fβ-hCG), combined with ultrasound measurements of fetal nuchal translucency and maternal age (Brizot et al. 1994). Recent advances have been made in the detection of fetal cells in maternal circulation as a non-invasive diagnostic tool for genetic abnormalities. Although a promising technique, more reliable detection rates would be required before this could be used as a diagnostic test (Bianchi, 2000).

1.3.1 Alpha-fetoprotein (AFP)

1.3.1.1 Structure, Localisation and Function

AFP is a 67-74 kDa protein that belongs to a superfamily of proteins including vitamin D and albumin. It has been shown to have homology in its primary, secondary and tertiary structure with serum albumin and, like albumin, has strong binding affinities with several ligands (Deutsch, 1991). The most notable binding molecules of AFP are polyunsaturated fatty acids, bilirubin, copper 2^+ and estrogens, although the biological relevance of this binding remains unclear. It has been suggested that AFP may be involved in transporting these substances within or to the fetus, perhaps to nourish fetal tissue (Deutsch, 1991). It has also been suggested that AFP has some regulatory functions over the maternal immune system, particularly immunosuppression (Ruoslahti and Hirai, 1978b). The similarities between AFP and serum albumin are also evident at the genetic level, with both having structurally related genes on chromosome 4. It is thought that the genes may have arisen from a common ancestral gene via gene divergence (Harper and Dugaiczyk, 1983). Three different forms of AFP have been isolated due to their varied ligand binding properties, caused by different glycosylation patterns. The different forms of AFP seem to be expressed by different tissues at various stages of gestation although all are detectable by immunological methods (Ruoslahti et al. 1978a).

AFP is found only at trace levels in healthy humans, however AFP may be detected in the serum of individuals who have certain medical conditions such as liver cirrhosis, hepatitis or liver, germ cell and gastrointestinal tumours. During pregnancy, AFP is detectable in the developing fetus, the AF and maternal serum. The main sites of AFP production in pregnancy are the yolk sac, fetal liver and fetal gastrointestinal tract (Ruoslahti and Hirai, 1978b).

1.3.1.2 AFP Concentrations in pregnancy

The maternal serum concentration of AFP is elevated during pregnancy. Concentrations begin to rise above non-pregnant levels at approximately ten weeks of gestation, and continue to do so until around 32 weeks of gestation, after which time they begin to decrease until term. AFP is also detectable in the AF, peaking at around fourteen weeks of gestation. AF levels of AFP are higher than those in maternal serum until just before parturition when the situation reverses (Seppälä and Ruoslahti, 1972; Ruoslahti and Hirai, 1978b; Wathen et al. 1991; Palomaki et al. 1993).

1.3.1.3 AFP levels in DS pregnancies

Several studies established that the maternal serum concentration of AFP in the second trimester of DS pregnancies is significantly lower than that in normal pregnancies. The DS multiple of the control median value (MoM) is around 0.75. (Cuckle et al. 1984; Fuhrmann et al. 1984; Merkatz et al. 1984; Tabor et al. 1984; Crandall et al. 1988; Spencer et al. 1992). It was demonstrated that measuring the maternal serum AFP concentration could be a useful method of selecting pregnancies for amniocentesis that have a high risk of being affected with DS, particularly when the AFP measurements were combined with maternal age (Cuckle et al. 1987; Tabor et al. 1987; Zeitune et al. 1991).

Studies of AF from DS pregnancies confirmed that the AFP level was significantly reduced, to a similar degree to that observed in maternal serum samples (Merkatz et al. 1984; Tabor et al. 1984; Cowchock and Ruch, 1984; Davis et al. 1985; Jones et al. 1986; Kaffe et al. 1988; Crandall et al. 1988; Zeitune et al. 1989). The MoM values reported in these studies range from 0.6 (Zeitune et al. 1989) to 0.734 (Jones et al. 1986) with a median of approximately 0.7MoM.

The AFP concentration has also been reported as being reduced in the maternal serum of first trimester DS pregnancies (Aitken et al. 1993; Fuhrmann et al. 1993; Spencer et al. 1994; Berry et al. 1995; Haddow et al. 1998). The reported AFP MoMs in the first trimester of DS pregnancies ranged from 0.62 to 0.97 with an average of 0.78MoM. There has, however, been some conflict of opinions as to whether first trimester screening using maternal serum AFP would be effective or not (Cuckle et al. 1988; Brock et al. 1990; Aitken et al. 1993; Crandall et al. 1993; Fuhrmann et al. 1993; Haddow et al. 1998).

In contrast to the situation in maternal serum and AF, one study has shown that endogenous placental concentrations of AFP are significantly higher in DS pregnancies (Newby et al. 1997). This study showed a greater than two fold elevation in AFP levels in placental extracts from DS pregnancies when compared to chromosomally normal pregnancies, suggesting altered placental transport of AFP in DS. Other placental abnormalities are associated with altered maternal serum AFP concentrations and it has been suggested that this may be due to increased leakage of AFP from sites of placental damage (Bendon, 1991).

1.3.2 Human Chorionic Gonadotrophin (hCG)

Maternal serum levels of the peptide hormone, human chorionic gonadotrophin (hCG), are elevated in DS pregnancies (Bogart et al. 1987). Elevated maternal serum levels of fβ-hCG have also been reported in the first and second (Macri et al. 1990; Spencer et al. 1992; Spencer et al. 1994) trimesters of DS pregnancies.

1.3.2.1 Structure and Localisation

hCG is a heterodimeric glycoprotein hormone that has an alpha and a beta subunit covalently bonded to each other. The 15kDa alpha subunit is common to other glycoprotein hormones such as follicle stimulating hormone (FSH), leutenising hormone (LH) and thyrotrophin stimulating hormone (TSH). Part of the untranslated sequences of the gene encoding this subunit also shows similarity to the human growth hormone genes (Fiddes and Goodman, 1981). In contrast, the 23kDa beta subunit is unique to hCG, thereby conferring the specificity of the hormone (Pierce and Parsons, 1981). There are four breakdown products of hCG that are found in AF, serum and urine along with ihCG (the active hormone). These breakdown products are nicked hCG

(hCG with a break in the β-subunit chain between residues 47 and 48 or 43 and 44), fβ-hCG, free α-hCG and nicked fβ-hCG (cut in the same positions as nicked hCG) (Cole et al. 1993). A fifth breakdown product of hCG called β-Core hCG (the terminally degraded product of the hormone), is detectable only in urine (Cole et al. 1997) and is the major form of hCG present in urine (Kato and Braunstein, 1988).

hCG is found in many tissues of the human body including the gonads, uterus, pituitary, digestive organs, lung, pancreas, placenta, fetal membranes and decidua (Braunstein et al. 1979). During the normal menstrual cycle and the early stages of pregnancy the corpus luteum is the primary site of hCG production. At around eight weeks of gestation, the placenta largely takes over the production of hCG. Immunohistochemical and RNA expression analysis of the placenta revealed that the predominant site of hCG α-subunit production is the cytotrophoblast, while β-subunit production is mainly localised to the syncytiotrophoblast (Hay, 1988; Kelly et al. 1991). Formation of intact hCG (ihCG) from the two subunits has been localised mainly to the syncytiotrophoblast layer of the placenta (Hay, 1988) although in the early stages of pregnancy the cytotrophoblast is capable of hCG production. This finding is confirmed by the report noting β subunit localisation in the cytotrophoblast cells of four to five week placentae, while from six weeks of gestation until term the syncytium is the primary site of β subunit localisation (Maruo et al. 1992). It is thought that the rate of hCG production is dependant on the rate of cytotrophoblast to syncytiotrophoblast differentiation (Hay, 1988). As well as the reported localisation of hCG to trophoblastic cells, the hCG β subunit has also been localised to Hofbauer cells (fetal macrophages) present in the villous stroma (Katabuchi et al. 1994).

1.3.2.2 Physiological actions of hCG

One of the main physiological actions of hCG in the early stages of pregnancy is to maintain the corpus luteum that otherwise would atrophy leading to the induction of menstruation. The corpus luteum plays an important role in the production of estrogens, progesterone, relaxin and inhibin during the early stages of pregnancy before the placenta and fetal membranes take over these functions. The roles of hCG in the later stages of pregnancy are largely unknown although it is thought to be involved in several processes including steroid biosynthesis, trophoblast differentiation, placental glycogen metabolism and placental function (Yen, 1991).

1.3.2.3 hCG levels in pregnancy

Soon after conception, an increase in the maternal serum and urine concentrations of hCG becomes apparent. This increase may be observed as early as six days after conception and detection of increased hCG concentrations in these fluids is used as an indication of pregnancy. The serum concentration of hCG continues to rise through the first trimester of pregnancy until a peak is reached at around eight weeks of gestation. The concentration of hCG then drops rapidly until approximately eighteen weeks of gestation when the decline halts and the lower hCG concentration is maintained for the remainder of pregnancy (Braunstein et al. 1976). The levels of hCG β subunit production largely mirror the trends in ihCG production while the rate of α subunit production increase throughout gestation until term. This indicates that production of the β subunit of hCG is the rate limiting step in ihCG synthesis (Vaitukaitis, 1974).

hCG is also detectable in the AF, although at a lower concentration than in the maternal circulation. The concentration of hCG in the AF peaks, at around 11 to 14 weeks, after which time the hCG concentration falls steeply to a low level which is maintained until parturition (Clements et al. 1976). The AF levels of both total hCG and fβ-hCG are higher in normal pregnancies where the fetus is female when compared with pregnancies when the fetus is male (Spencer et al. 1997a).

Relative levels of ihCG, α-hCG and fβ-hCG vary in the placenta at different stages of gestation. In the first trimester of pregnancy, the concentrations of all forms of hCG are high with a peak observed at around 7-10 weeks. The total hCG levels then drop until parturition. In the third trimester of pregnancy the concentration of α-hCG remains at a similar level to that observed in the second trimester, while the levels of both β-hCG and ihCG continue to decline. This results in the alpha subunit level exceeding that of the beta and intact forms of hCG in the third trimester of pregnancy (Vaitukaitis, 1974).

1.3.2.4 hCG Levels in DS pregnancies

Numerous studies have confirmed that the second trimester maternal serum levels of both intact hCG and fβ-hCG are elevated to around twice the normal MoM value in DS pregnancies (Bogart et al. 1987; Wald et al. 1988b; Macri et al. 1990; Nørgaard-Pedersen et al. 1990; Crossley et al. 1991; Haddow et al. 1992; Ryall et al. 1992; Spencer et al. 1992; Stone et al. 1993). A *meta* analysis reported a DS maternal serum

MoM of 1.83 for fβhCG and 1.29 for ihCG (Wald et al. 1997b). The large elevation observed in DS pregnancies make hCG the best second trimester serum marker for affected pregnancies, giving a detection rate of 30%, for a 3% false positive rate. Consequently, fβ-hCG or ihCG measurements are included in almost all biochemical screening programmes (Wald et al. 1996). It has been reported that the use of fβ-hCG in combined screening programmes provides a better detection rate of DS pregnancies compared with the use of ihCG with an approximately 10% increase in sensitivity (Macri et al. 1990; Spencer, 1991; Macri et al. 1994; Wald et al. 1997a). One study reported no difference in detection rate but a decreased false positive rate with the use of fβ-hCG in a combined screening programme for DS (Stone et al. 1993). Some studies have reported an increase in the maternal serum levels of free α-hCG in the second trimester of DS pregnancies (Bogart et al. 1987; Bogart et al. 1989; Ryall et al. 1992). Others failed to find any increase in the free α-hCG levels in maternal serum (Spencer, 1993).

In the first trimester of DS pregnancies, the maternal serum level of fβ-hCG is significantly elevated to almost twice the control level and is used in first trimester screening for DS (Ozturk et al. 1990; Aitken et al. 1993; Macri et al. 1993; Spencer et al. 1994; Haddow et al. 1998). The first trimester levels of ihCG were shown to be slightly elevated by some studies, whilst others found no significant increase in DS pregnancies (Cuckle et al. 1988; Brock et al. 1990; Aitken et al. 1993; Crandall et al. 1993; Brizot et al. 1995). Some studies have looked at the first trimester concentrations of maternal serum free α-hCG in DS pregnancies. One study found a slight increase (Ozturk et al. 1990), while another noted no significant difference from normal levels (Bogart et al. 1989). In contrast, Kratzer *et al* (1991) reported a significant decrease in the maternal serum levels of the alpha subunit. Table 1.3.2.4 summarises the average MoM levels of the different forms of hCG in first trimester DS pregnancies.

The AF of DS pregnancies was shown to have significantly increased levels of ihCG, fβ-hCG and total hCG (Newby et al. 1997; Spencer et al. 1997a). The degree of elevation in the different forms of hCG reported in these studies was slightly lower than the degree of elevation observed in maternal serum in the second trimester of DS pregnancies (table 1.3.2.4).

Maternal urine concentrations of fβ-hCG and β-core hCG have been studied in DS pregnancies. Urinary fβ-hCG levels in both the first (Spencer et al. 1997b) and second trimesters (Spencer et al. 1996; Hayashi et al. 1996) of DS pregnancies are significantly elevated. The degree of elevation in fβ-hCG levels in urine is more in the second trimester (table 1.3.2.4). It was suggested in these studies that this marker could be used in the detection of DS pregnancies in either trimester of pregnancy. Maternal urine β-core hCG levels are elevated to over three times the normal level in the second trimester of DS pregnancies (Cuckle et al. 1994; Hayashi and Kozu, 1995; Canick et al. 1995; Spencer et al. 1996; Isozaki et al. 1997; Bahdo-Singh et al. 1999; Cuckle et al. 1999). Only one study found significantly elevated first trimester levels of β-core hCG in DS pregnancies (Spencer et al. 1997b) with others indicating no difference in the first trimester levels of this protein (Kornman et al. 1997; Macintosh et al. 1997). It has been suggested that β-core hCG may be a useful screening marker for DS in the second trimester of pregnancy, however the lack of availability of an optimised assay for this protein in urine complicates its use in routine screening (Cole et al. 1997).

A study using placental homogenates from control and DS pregnancies has shown that the placental levels of both fβ-hCG and ihCG are subject to a three to four fold increase in DS pregnancies, mirroring the situation in maternal serum, urine and AF (Newby et al. 1997).

	1st Trimester		2nd Trimester		
	serum	**urine**	**serum**	**AF**	**urine**
ihCG	1.29*		2.06*	1.38	2.30
fβhCG	1.83*	1.81	2.20*	1.77	2.55
αhCG	1.00*		1.43*		
β-core hCG	NP	3.08	NP	NP	3.67*

Table 1.3.2.4 *Summary of average DS median MoM values for each form of hCG studied in maternal serum, AF and maternal urine in the first and second trimesters of pregnancy. NP=not present in that fluid. *Indicates MoM values reported in the meta analysis by Wald et al (1997b). Others are calculated from the references cited in the body text of section 1.3.2.4.*

1.3.3 Unconjugated Estriol (UE_3)

Unconjugated estriol (UE_3) is a pregnancy associated steroid hormone synthesised in the placenta from precursors derived from the fetus. Low total estriol levels were first noted in the maternal urine of DS pregnancies (Jørgensen and Trolle, 1972). Later work indicated that lower than normal maternal serum levels of UE_3 were associated with DS pregnancies (Canick et al. 1988). A *meta* analysis of several studies looking at second trimester UE_3 levels in DS pregnancies gave a median DS median MoM of 0.72 based on 733 affected pregnancies (Wald et al. 1997b). It was suggested that this measurement be combined with AFP levels, hCG levels and maternal age to further improve the accuracy of prenatal screening tests (Canick et al. 1988; Wald et al. 1988a; Wald et al. 1988b). The use of these three biochemical markers in combination with maternal age is commonly called the "triple test". Wald (1998a) suggested that not only would the detection rate be improved if these combined tests were adopted, but the false positive rate would also be reduced. The value of UE_3 as a first trimester screening marker has also been studied (Cuckle et al. 1988) and it was found that levels were significantly lower in the first trimester. The average reduction observed in a series of studies looking at 210 DS pregnancies was 0.71MoM (Wald et al. 1997b). It was suggested that UE_3, when used in combination with other markers, would form part of a useful screening programme for DS in the first trimester (Cuckle et al. 1988; Crandall et al. 1993).

These observations were not supported by other studies carried out in the first and second trimesters of pregnancy. Despite reporting a significant reduction in the level of UE_3 in both trimesters, these studies could not find any significant improvement in detection rates by combining these results with age, AFP and either ihCG or fβhCG in prenatal screening programmes. This was because the change in UE_3 concentration is correlated with the change in AFP concentration meaning alterations in the levels of UE_3 in DS pregnancies merely reflect the changes seen in AFP levels (Crossley et al. 1993; Aitken et al. 1993; Wenstrom et al. 1997; Haddow et al. 1998).

1.3.4 Inhibin-A

Several studies have reported a significant increase in the second trimester levels of the dimeric glycoprotein inhibin-A in maternal serum from women with DS pregnancies, when compared with the serum levels from apparently normal pregnancies (Aitken et al.

1996; Wald et al. 1996; Cuckle et al. 1996; Wallace et al. 1996; Lambert-Messerlian et al. 1996a; Lambert-Messerlian et al. 1998). The DS level reported by these studies ranges from 1.62MoM (Cuckle et al. 1996) to 2.6MoM (Wallace et al. 1996). It has been suggested that inhibin-A be included in the second trimester prenatal screening programme for DS as it may increase the detection rate currently achieved when measuring hCG and AFP levels in maternal serum by approximately 10% (Cuckle et al. 1996). In the first trimester inhibin-A levels have been reported as being significantly increased to 2.46MoM in one study (Wallace et al. 1995) while a different study reported no significant difference from the normal situation with a DS median MoM of 1.38 (Aitken et al. 1996). A study by Wallace *et al.* (1997) discovered a significantly decreased level of inhibin-A in AF collected from DS pregnancies. This group reported that the median DS MoM was significantly decreased to 0.77 MoM, the opposite trend from that observed in the maternal serum.

1.3.5 Superoxide Dismutase (SOD1)

None of the maternal serum screening markers described in previous sections has their gene on chromosome 21. One chromosome 21 specific marker, which has been evaluated as a potential screening marker, is superoxide dismutase (SOD1). Increased SOD 1 activity has been observed in fetal erythrocytes (Portsmann et al. 1990), AF (Baeteman et al. 1985), maternal serum (Ognibene et al. 1999) and maternal blood (Cuckle and Arbuzova, 2000) from DS pregnancies. In general, this increase in activity was by a factor of 1.5 as would be expected for the increased gene dosage effect. The overlap between control and DS levels of activity indicates that this may not be a useful screening marker, although the number of cases on which these studies were based is limited. Elevated activity has also been reported in the tissues of DS individuals as would be expected for a gene expressed on chromosome 21 (Sinet, 1982). This elevated activity has been widely studied in the brains of DS patients where it is thought to play a key role in neuronal degradation leading to neural dysfunction and early onset Alzheimer's type dementia (Iannello et al. 1999).

SOD1 is a copper/zinc containing homodimeric enzyme with each dimer having a mass of 15kDa. It is present in the cytosol of cells where it catalyses a reaction that destroys free radicals produced during normal cellular reactions. These free radicals would otherwise be toxic to biological systems. The reaction takes two superoxide radicals and

two protons and converts them into oxygen and hydrogen peroxide. The hydrogen peroxide is then metabolised to water by either glutathione peroxidase or catalase. Although this reaction is protective against oxidative damage caused by superoxide radicals, excess SOD1 activity could lead to accumulation of hydrogen peroxide in the cell if glutathione peroxidase or catalase do not upregulate to clear the extra hydrogen peroxide. Hydrogen peroxide is toxic to cells and can also react with superoxide radicals to give rise to the more dangerous hydroxyl radical leading to more oxidative damage. The extra copy of this gene in DS pregnancies could therefore have a bearing on the pathophysiology observed in these pregnancies.

1.3.6 Pregnancy Associated Plasma Protein A (PAPP-A)

Lower than normal levels of placental derived PAPP-A have been noted in the first trimester of DS pregnancies. It was recently reported that PAPP-A is infact insulin like growth factor binding protein-4 (IGFBP-4) protease (Conover et al. 2001; Byun et al. 2001). By cleaving IGFBP-4, PAPP-A enhances the activity of insulin like growth factor because less IGFBP-4 is present to attenuate the activity of IGF. Levels of PAPP-A are less than half that in unaffected pregnancies (Wald et al. 1992; Spencer et al. 1994; Brizot et al. 1994; Berry et al. 1997). A *meta* analysis of twelve studies reported an average DS median MoM of 0.38. Unlike hCG, this marker is only suitable for use in the first trimester of pregnancy because DS levels are normal in the second trimester of pregnancy (Berry et al. 1997; Wald et al. 1997b). For first trimester screening protocols PAPP-A levels are combined with fβ-hCG levels, an ultrasound measurement of nuchal translucency and maternal age to give a risk of DS pregnancy (Wald et al. 1997b).

1.3.7 Other Maternal Serum Markers of DS

Many other potential maternal serum markers of DS pregnancies have been investigated in both the first and second trimesters of pregnancy (table 1.3.7). Despite the majority of these markers proving to be of little clinical use in the detection of DS pregnancies, many of these markers are of placental origin or can influence placental growth suggesting altered function of the placenta in DS pregnancies.

Marker	1st Trimester MoM	2nd Trimester MoM	References
URNAP		1.65MoM (76)	(Wald et al. 1997b)
SP-1	0.81MoM (111)	1.47MoM (379)	(Wald et al. 1997b)
α Inhibin	1.27MoM (34)	1.63MoM (64)	(Wald et al. 1997b)
Activin-A	1.36MoM (45)	1.04MoM (50)	(Lambert-Messerlian et al. 1998; Cuckle et al. 1999; Spencer et al. 2001b)
Pro MBP	0.73MoM (15)	1.62MoM (105)	(Christiansen et al. 1999)
p43		1.58MoM (42)	(Moroz et al. 2000)
IGFBP-3		1.09MoM (9)	(Chu et al. 1998)
Ca 125	1.14MoM (34)	0.94MoM (81)	(Wald et al. 1997b)
PLGF	1.26MoM (45)	0.67MoM (24)	(Debieve et al. 2001; Spencer et al. 2001a)

Table 1.3.7 *Summary of other suggested maternal serum markers of DS in first and second trimesters of pregnancy. URNAP=urea resistant neutrophil alkaline phosphatase SP-1=schwangerschafts protein 1, Pro MBP=pro eosinophil major basic protein, p43=placental isoferritin p43 component, IGFBP-3=insulin like growth factor binding protein-3, Ca 125=cancer antigen 125, PLGF=placental growth factor. Number of DS pregnancies on which MoM values are based is indicated in parentheses. Where possible MoM values are those reported in meta analyses.*

1.3.8 Why are Maternal Serum Marker Levels Altered?

Despite the work carried out to determine the degree of variation in maternal serum and maternal urine marker levels between normal and DS pregnancies, little is understood about why these levels are altered. These alterations and the reasons behind them have provoked some interest (Canick et al. 1988; Chard, 1991a; Waller et al. 1993; Knöfler, 1999; Goshen, 1999) and some studies of the mechanisms that control the synthesis and release of these markers have been carried out to try and further understand normal production as well as the reasons behind the altered marker levels in abnormal pregnancies. These studies have mainly focused on differences in placental function in chromosomally abnormal pregnancies when compared to controls.

1.4 Placenta

1.4.1 Placental development and function

The human placenta is a unique organ that regulates the transfer of nutrients, hormones, waste products, oxygen and other soluble factors between the mother and fetus. The placenta produces many protein and steroid hormones, including hCG, progesterone, human placental lactogen (hPL), chorionic thyrotrophin, human chorionic follicle stimulating hormone, human uterotropic placental hormone and chorionic corticotrophin. The placenta also produces estrogens with assistance from the fetal adrenal gland and liver (Osathanondh and Tulchinsky, 1980).

The placenta is formed of maternal and fetal components. The maternal component is derived from the decidua, while the fetal part arises from the villous chorion. Five days after fertilisation, the cell mass changes to a hollow structure called the blastocyst. The blastocyst consists of an inner cell mass, which will later form the fetus, and an outer shell called the chorion. The chorion is composed of trophoblast cells and will later form part of the placenta. Seven to twelve days following fertilisation, the blastocyst implants in the endometrium during which time the chorion becomes a thick double-layered structure. The inner cell layer is composed of cytotrophoblast cells, which are enclosed in the outer syncytial layer. The cytotrophoblast cells rapidly proliferate causing the formation of finger like projections of trophoblast cells called primary villi that project into the intervillous space. The formation of villi begins at around four weeks of gestation, with primary villi consisting of only cytotrophoblast cells clothed in an outer syncytial layer. During the following week of gestation, the villi begin to develop a mesenchymal core and become branched. They are now termed secondary villi. At the end of the fifth week of gestation tertiary villi, which are so called because of the presence of vasculature, can be found. As pregnancy proceeds tertiary villi predominate, with the formation of branched villi continuing until term (Moore and Persaud, 1998; Kingdom and Kaufmann, 2000).

Initially, the villi form in a hypoxic environment and receive nutrients from uterine glandular secretions and plasma filtrate in the intervillous space rather than the maternal circulation. It is thought that this oxygen poor environment minimises damage to the developing fetus by oxidative stress (Jaffe et al. 1997; Burton et al. 1999). The onset of maternal circulation into the intervillous space is thought to occur at around ten to

twelve weeks of gestation. This event greatly increases the oxygen tension from <20mmHg at eight to ten weeks to >50mmHg at 12 weeks. The fetus now receives nutrients and oxygen from the maternal circulation (Rodesch et al. 1992). The fetal circulation (poorly oxygenated and low in nutrients) enters the formed placenta via the umbilical arteries and continues into the branched villi where exchange of nutrients, waste, oxygen and carbon dioxide occurs across the trophoblastic layer. The syncytiotrophoblast is bathed in maternal blood (rich in oxygen and nutrients), which enters the intervillous space via the endometrial spiral arteries and then drains back into the maternal circulation via the endometrial veins after an exchange of oxygen and nutrients through the trophoblast cells, has occurred with the fetal circulation. The oxygenated fetal blood then circulates from the villi back to the developing fetus via the umbilical vein (see figure 1.4.1) (Moore and Persaud, 1998). To meet the increased demand for maternal blood to the placenta, maternal endometrial spiral arteries must undergo physiological remodelling which involves removal of the spiral artery smooth muscle. This enlarges the blood vessels and diminishes their sensitivity to vasoconstrictors thereby allowing increased blood flow. This process is mediated by the invasion of extravillous cytotrophoblasts (EVT).

1.4.2 Trophoblast Cells

All maternal-fetal exchange occurs over the trophoblastic layer making these cells of particular interest when studying placental physiology. They are also the site of production of most pregnancy associated hormones and proteins including hCG. This layer consists of the inner cytotrophoblast cells, which differentiate to form the outer continual syncytial layer (Boyd and Hamilton, 1970).

1.4.2.1 Cytotrophoblast cells

The inner cytotrophoblast layer consists of cells with a uniform oval shape, a clear cytoplasm and a large, single, hyperchromatic nucleus. They have distinct cell membranes and have the general appearance of epithelial cells (Boyd and Hamilton, 1970; Mazur and Kurman, 1989). Cytotrophoblast cells are the precursor cells that differentiate to form the syncytiotrophoblast layer (Carter, 1964). In the early placenta an almost continual layer of these cells can be observed below the syncytiotrophoblast while by term very few residual cytotrophoblast cells remain. Many factors influence the differentiation of cytotrophoblast cells including growth factors and cytokines. The

process of syncytialisation has been associated with the production of various placental factors including hCG and hPL both of which have been used as *in vitro* markers of the presence of viable trophoblast cells (Kliman and Feinberg, 1992).

During early pregnancy, cytotrophoblast cells also break through the syncytial layer and form cell columns that penetrate the uterine lining. Villi that have cell columns at the tip are called anchoring villi because they form a physical connection between the placenta and the decidualised endometrium. cytotrophoblast cells, derived from cell columns, invade the spiral arteries and are thought to induce physiological remodelling. These EVT can invade the lumen of spiral arteries directly and migrate deeper into the endometrium. As they migrate the EVT replace the endothelial cells of the spiral arteries causing the remodelling required for increased blood flow. This process is likely to begin as early as four to six weeks of gestation. EVT also invade the decidua and progress into inner third of the myometrium where they cluster around the spiral arteries. EVT can also fuse to form multinucleated giant cells (Lyall and Robson, 2000).

1.4.2.2 Syncytiotrophoblast

The syncytiotrophoblast forms the outer surface of the placenta, which is bathed in maternal blood. It is a continual cellular layer with no membranes separating cells and has dense, pyknotic nuclei. Electron microscopy has revealed that the syncytiotrophoblast has many microvilli that increase the surface area where exchange between mother and fetus occurs. In the third trimester, nuclei in the syncytiotrophoblast aggregate to form bud like structures called syncytial knots that protrude from the syncytium. These knots detach from the villi and enter the maternal circulation (Boyd and Hamilton, 1970). Placental alkaline phosphatase (PLAP) is produced by the syncytium and can be used as an *in vitro* marker of the presence of syncytium.

1.4.3 Villous Morphology

When studied microscopically, different cell types that form the villous structure can be identified. This morphology alters as pregnancy progresses (see figure 1.4.3). Early in pregnancy, the villi are smaller and the fetal blood vessels are small and centrally located. There is also a clear layer of individual cytotrophoblast cells underlying the continual syncytial layer. As pregnancy proceeds, the villi increase in size and the

endothelium can often be separated from the maternal circulation only by the syncytial layer. The number of cytotrophoblast cells becomes fewer as pregnancy proceeds and fibrinoid deposits are visible in areas of trophoblastic damage. The villous stroma or core is mainly a loose network of fibroblast cells with Hofbauer cells (fetal macrophages) also found within this area (Boyd and Hamilton, 1970; Moore and Persaud, 1998).

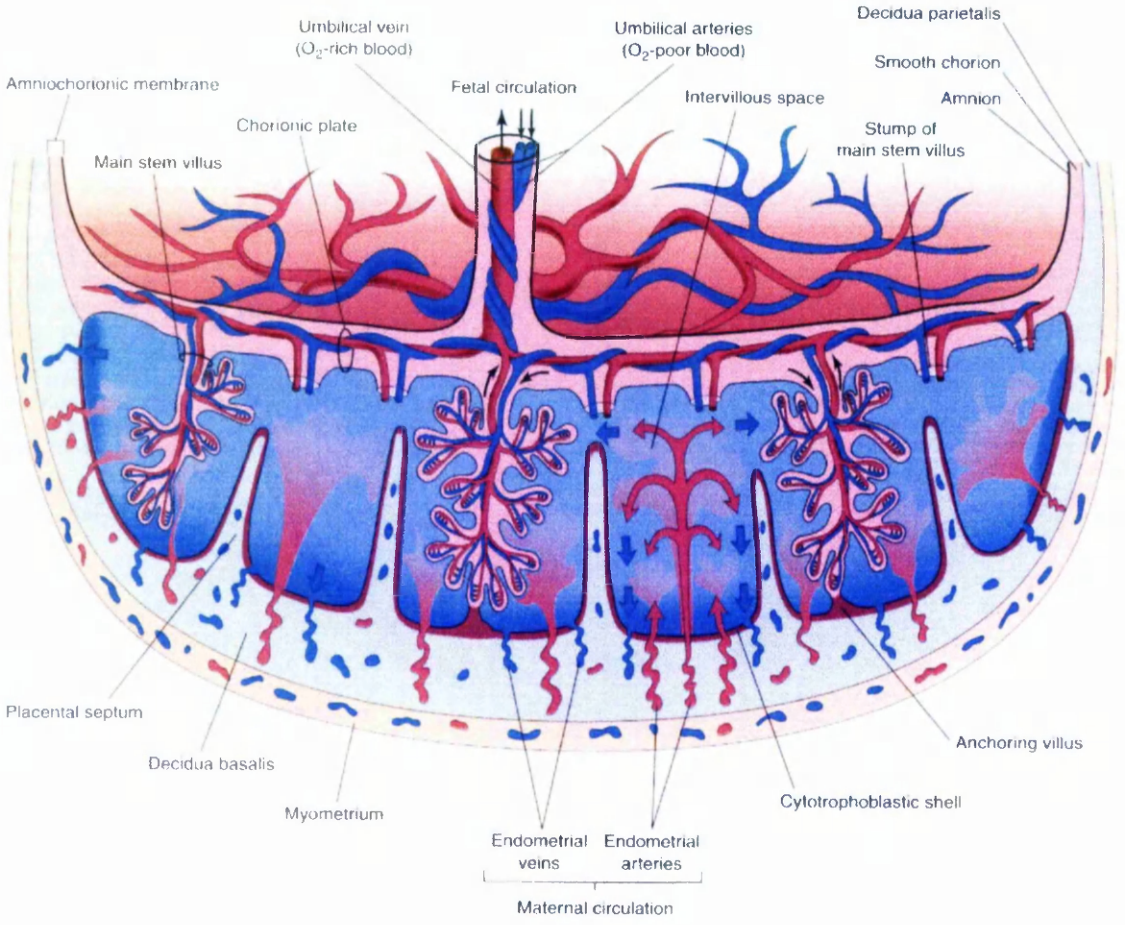


Figure 1.4.1 *Diagrammatic representation of a term placental cross section. This illustrates the interaction between the maternal (decidua & myometrium) and fetal (villous chorion) sections of the placenta. Arrows indicate the direction of maternal and fetal blood flow, with oxygenated blood shown in red and oxygen-poor blood shown in blue. Reproduced from Moore and Persaud (1998) in Before We Are Born with kind permission from WB Saunders Company.*

1.4.4 Placental Morphology in Abnormal Pregnancy

Several groups have studied placental size and morphology in trisomic pregnancies, as well as the proliferation and apoptosis of placental cells. This has uncovered several abnormalities in the placental structure of these pregnancies.

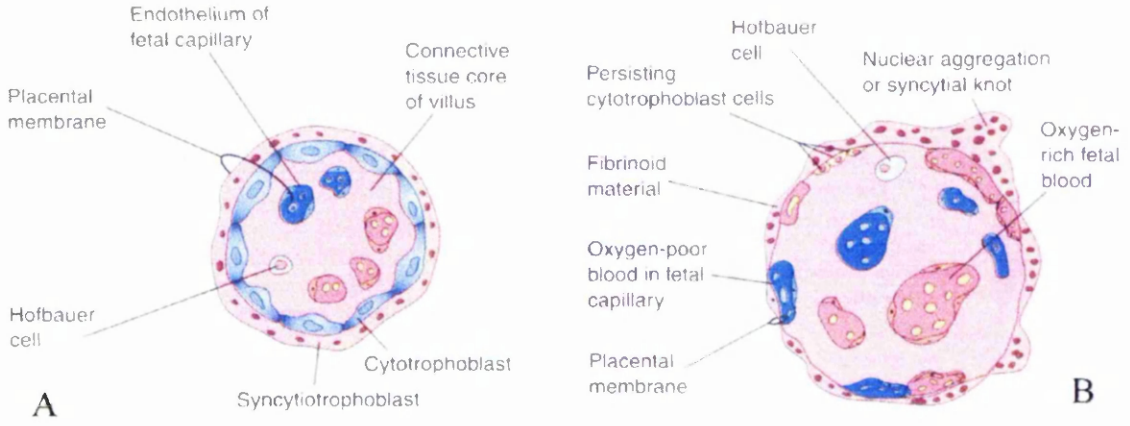


Figure 1.4.3 *Diagram of villous cross section in first trimester (A) and term (B) placentae. Reproduced from Moore and Persaud (1998) in Before We Are Born with kind permission from WB Saunders Company.*

1.4.4.1 Placental & Fetal Size in DS

Early studies (MacAfee et al. 1970; Kučera, 1971) measured the weight and size of a large number of DS placentae and fetuses and found no significant difference from the normal gestational average. Two smaller studies found evidence of either normal or slightly heavier placentae in DS pregnancies (Arizawa and Nakayama, 1992; Qureshi et al. 1997). Ultrasound studies of the crown-rump length and biparietal diameter of DS fetuses have also shown them to be of the appropriate gestational size (Wald et al. 1993; Kuhn et al. 1995).

1.4.4.2 Placental Morphology in DS

Several studies have noted considerable morphological abnormalities in trisomic placentae when studied at a microscopic level. Table 1.4.4.2 outlines these abnormalities and the different studies that have noted them. These morphological abnormalities could potentially play a role in the altered levels of hCG secreted into maternal serum during DS pregnancies. Analysis of placental cells from trisomic pregnancies by flow cytometry found no significant difference in the number of apoptotic cells in second trimester trisomic placentae when compared with gestation matched controls (Halperin et al. 2000).

Histological Anomaly	Abnormality	References
Trophoblast Hypoplasia	T21, T18	(Rochelson et al. 1990; Jauniaux and Hustin, 1998)
Stromal Oedema	T21, T18	(Rochelson et al. 1990; Jauniaux and Hustin, 1998)
Reduced Vascularisation	T21, T18, T13	(Oberweis et al. 1983; Rochelson et al. 1990; Qureshi et al. 1997; Jauniaux and Hustin, 1998; Roberts et al. 2000)
Irregular Villous Maturation	T21, T18, T13	(Oberweis et al. 1983; Rochelson et al. 1990; Qureshi et al. 1997)
Immature Villi	T21, T18, T13	(Oberweis et al. 1983; Rochelson et al. 1990; Arizawa and Nakayama, 1992; Roberts et al. 2000)
Loose Stroma	T21, T18, T13	(Oberweis et al. 1983; Qureshi et al. 1997; Jauniaux and Hustin, 1998)
Persistence of Haufbauer Cells	T21, T18, T13	(Oberweis et al. 1983)
Reduced Syncytialisation	T21, T18, T13	(Oberweis et al. 1983)
Persistence of cytotrophoblast cells	T21	(Roberts et al. 2000)

Table 1.4.4.2. *Histological anomalies noted in trisomic pregnancies by various studies. T21=trisomy 21, T18=trisomy 18, T13=trisomy13.*

1.4.4.3 Placental Tissue Levels of DS Serum Markers

A study by Newby *et al* (1997) looked at levels of various maternal serum markers of DS in placental tissue extracts obtained from 2nd trimester pregnancies. Both ihCG and fβ-hCG were significantly elevated in placentae from DS pregnancies, mirroring the increase observed in maternal serum. In contrast, placental AFP levels were significantly increased in placentae from DS pregnancies, the opposite of the reduction in AFP levels observed in maternal serum. This group also studied the reason behind decreased UE_3 levels in maternal serum of DS pregnancies. It was found that UE_3 was significantly reduced in the placenta, as were levels of the precursor dehydroepianrosterone sulphate (DHEAS). This suggested that reduced serum levels of UE_3 could be due to reduced synthesis in the placenta because of a diminished supply of the precursor DHEAS (Newby et al. 2000).

A study, which supports previous findings on increased β-hCG subunit protein concentration in DS placentae, also reported increased LH/hCG receptor expression in DS placentae from 12-16 weeks of gestation. This was carried out by comparing immunohistochemical and *in situ* hybridisation staining intensity between DS and control placental sections. LH/hCG receptors and receptor mRNA were primarily localised to the syncytiotrophoblast layer with weaker staining of the cytotrophoblast cells. This study did not find a significant difference in free α or ihCG in DS placentae, although there was a trend towards increased ihCG (Jauniaux et al. 2000). A study of first trimester and early second trimester placental mRNA from control and DS placentae, by northern blot analysis, revealed no significant difference in either α-hCG or β-hCG mRNA levels between the groups (Brizot et al. 1995). One group reported reduced mRNA expression of the syncytialisation markers hPL, hCGα, hCGβ, placental growth hormone and leptin in DS placental mRNA extracts (Frendo et al. 2000; Evain-Brion et al. 2000). They suggested that *in vivo* formation of syncytiotrophoblast is defective in DS pregnancies.

1.4.5 Trophoblast cell culture

To further understand the mechanisms behind increased maternal serum hCG in DS pregnancies, it would be useful to use an *in vitro* model to study placental production of this hormone. Since the placental trophoblast cells are the major site of hCG synthesis, culturing these cells would be of particular use for studying placental production of hCG in either normal or pathological pregnancies.

1.4.5.1 Trophoblast Isolation

Several groups have developed techniques to culture placental trophoblast cells *in vitro* (Kliman et al. 1986; Bax et al. 1989; Bloxam, 1991; Bloxam et al. 1997a; Bloxam et al. 1997b). The culture methods used by these groups employ different methods of isolating mono-nuclear cytotrophoblast cells and culturing these in vitro to allow them to differentiate into a continual syncytial layer. The Kliman method (Kliman et al. 1986) involves the dissection and mincing of healthy villous tissue from a fresh placenta. The tissue is then serially digested using trypsin and deoxyribonucease (DNase) whilst shaking to release the cytotrophoblast cells. The supernatant containing the cytotrophoblast cells is collected and the trypsin inactivated by spinning the cells through calf serum. The cells are then centrifuged through a Percoll gradient to isolate

the mononuclear cytotrophoblast cells. The Bax method (Bax et al. 1989) involves the dissection of villous tissue, which is digested using dispase and pancreatin to loosen trophoblast cells. The tissue is transferred to a different tube containing buffered saline and agitated to dislodge the cytotrophoblast cells. Fragments of villous tissue in the resulting cell solution are strained from the cells using layers of gauze. The cells are then spun through a Percoll gradient to isolate the mononuclear cytotrophoblast cells. Both of these methods are routinely employed to isolate placental cytotrophoblast cells.

1.4.5.2 Trophoblast Purification and Quality Control

First trimester placental trophoblasts require further purification due to the high level of contamination in these isolations. Term trophoblast isolations may also require further purification, depending on experimental requirements, because the 90-95% purity of cells obtained after Percoll gradient purification may not be adequate. Further immunopurification protocols have been employed by various groups using different antibodies to remove contaminating cells from trophoblast preparations. One method is to use magnetic beads coated with anti-HLA class I antibody. Villous trophoblast cells do not express the HLA-Class I major histocompatibility antigens, while other placental and maternal cell types express these antigens. This makes using anti-HLA class I antibodies a good method of removing contaminating cells from trophoblast isolations (Kawata et al. 1984; Butterworth and Loke, 1985). One group (Morrish et al. 1991b), who observed that all placental cells except trophoblasts express the CD9 antigen used magnetic beads coated with anti CD9 antibodies to remove contaminating cells.

Regardless of the method of isolation and purification, it is vital to ensure that the correct cells have been isolated. This can be achieved by analysing the isolated cells by immunocytochemistry using a panel of antibodies specific for various contaminating cells and confirming the presence of cytotrophoblast cells using cytokeratin antibodies. Cytokeratin is a well established marker of epithelial cells. It is a filamentous protein found in the cell membrane usually associated with desmosomes and hemidesmosomes, and gives structural support to epithelial cells. Recently it was shown that cytokeratin 7 in particular is the best trophoblast specific marker because other non-trophoblastic placental cells have been shown to express other forms of cytokeratin (Haigh et al. 1999). Trophoblast cells do not express vimentin, another filamentous protein that is typically expressed by mesenchymal and endothelial cells. Consequently, most contaminating cells found in trophoblast preparations show positive reactivity with

antibodies targeted against vimentin. Other antibodies targeted against specific cells that could potentially contaminate trophoblast cultures may be used to assess the primary contaminants of a particular isolation protocol (table 1.4.5.2).

Antigen	Clone	Reactivity in Placenta	Reference
CD 9	P1/33/2	Fibroblasts, EVT, maternal leukocytes, endothelial cells	(Morrish et al. 1991b; Blaschitz et al. 2000)
CD 45	2B11 & PD7/26	Maternal leukocytes; fetal leukocytes & Hofbauer cells weak	(Blaschitz et al. 2000)
CD163	Ber-MAC 3	Hofbauer cells	(Blaschitz et al. 2000)
Cytokeratin 7	OV-TL 12/30	All trophoblast cells	(Haigh et al. 1999; Blaschitz et al. 2000)
Fibroblast specific antigen (FSA)	ASO2	Fibroblasts, endothelial cells	(Blaschitz et al. 2000)
HLA Class I	W6/32	All cells except villous trophoblast	(Kawata et al. 1984; Butterworth and Loke, 1985)
Vimentin	V9	Maternal & fetal leukocytes, fibroblasts, endothelial cells, Hofbauer cells	(Blaschitz et al. 2000)

Table 1.4.5.2 *Reactivity of each antibody used for screening placental trophoblast cell isolations and the reference citing the use of the antibody.*

1.4.5.3 Trophoblast Cell Lines

There have been many studies using trophoblast cell lines as a model of normal trophoblast function. These cell lines are more convenient to use than primary cultures because they do not require a lengthy isolation protocol and the number of cells is unlimited unlike the limited yield obtained from an isolation of primary cells from a single placenta. Cell lines are usually much easier to culture than primary cells making them an attractive alternative to primary cell cultures. The most commonly used cell

lines are BeWo, JEG and JAR, which are all derived from choriocarcinomas, however there are many other cell lines that have arisen by viral transformation or fusion of normal cytotrophoblast cells with choriocarcinoma cells (reviewed by King *et* al (2000)). Workshops at the European Placental Group conference in Schladming (1999) (King et al. 2000) and at the Rochester Trophoblast Conference (2000) (Shiverick et al. 2001) have outlined criteria for evaluation of these cell lines. It was suggested that villous trophoblast cell lines should be positive for cytokeratin 7 and be negative for HLA-class I and CD9, while extravillous cell lines should be positive for cytokeratin 7, HLA-G and CD9 (King et al. 2000; Shiverick et al. 2001). The excretion of hCG and hPL from cultures is also used as an indication of trophoblast cell origin. The suitability of this and HLA-G and cytokeratin 7 as trophoblast markers has been called into question because tumour cells also express these markers and can excrete hCG and hPL. It was therefore concluded that a broad panel of markers, similar to those used for characterisation of primary cytotrophoblast preparations, be employed when evaluating the validity of trophoblast cell lines.

Recently there has been some doubt as to the validity of these cell lines, in particular if they are suitable models of normal trophoblast cells. A clear illustration of the importance of rigorous characterisation of trophoblast cell lines is the recent case of the widely used ED_{27} trophoblast cell line. This cell line was isolated from non-malignant first trimester chorionic villi. It exhibited many traits of trophoblast cells including PLAP, hPL, HLA-G and cytokeratin expression and the ability to synthesise estrogens and a degree of syncytialisation. In contrast to the situation in primary trophoblast cultures ED_{27} cells do not secrete hCG during the first days of culture and HLA-Class I antigens are expressed on the cell surface. Due to these discrepancies and differences in experimental results obtained using ED_{27} cells and primary trophoblast cultures, the validity of this cell line was further investigated and it was discovered that it displayed molecular markers of HeLa and WISH cell lines. It was therefore suggested that ED_{27} cells had, at some point, been contaminated with these cell types (Kniss et al. 2001). This discovery means that published work using ED_{27} cell lines, and possibly other cell lines which have not undergone such rigorous characterisation, as a model of normal trophoblast function have to be interpreted with caution.

1.4.5.4 Culture of Trophoblast Cells from DS Placentae

Most culture systems have studied the growth and function of normal trophoblastic cells, although two groups have studied isolated trophoblast cells from DS pregnancies (Eldar-Geva et al. 1995; Frendo et al. 2000; Evain-Brion et al. 2000; Frendo et al. 2001). The first group studied four normal and three DS trophoblast cultures from 2nd trimester terminated pregnancies. The release of hCG into the culture medium was measured and morphological changes in the cells were monitored by light microscopy. The hCG mRNA levels were also studied in one DS and one control culture at 24h after plating. The quantity of hCG secreted from the cells, taken as a ratio of protein content, was significantly higher in the DS cultures, as was the quantity of hCG mRNA levels isolated from the single culture. It was observed that the quantity of hCG secreted from DS cultures from 2nd trimester placentae resembled the hCG secretion levels from normal 1st trimester cultures. The DS cultures did, however, appear to syncytialise less efficiently than the control cultures, and the number of cells adhering to the culture surface was also less in the DS cultures as measured by total protein content of lysed cells remaining on the culture surface.

The second group has studied various aspects of DS trophoblast function in culture. It was found in both reports by the group that these cells failed to syncytialise as control trophoblast cells did, and less hCG was produced by DS cultures (Frendo et al. 2000; Evain-Brion et al. 2000). These results were based, in the former report, on 8 control and 8 DS placentae collected from second trimester terminations (Evain-Brion et al. 2000). The second report was based on 10 control and 15 DS placentae collected from both the second and third trimesters (Frendo et al. 2000). They suggested these *in vitro* observations supported the report of persistence of cytotrophoblast cells observed in sections of chorionic villi collected from DS placentae (Roberts et al. 2000).

The same group suggested that this failure to syncytialise was due to overexpression of the chromosome 21 product, copper zinc superoxide dismutase (SOD-1) (Evain-Brion et al. 2000; Frendo et al. 2001). Both reports indicated that DS placentae expressed higher levels of SOD-1 than gestation matched controls. They also transfected normal term trophoblast cells with a plasmid containing the SOD-1 gene that caused increased SOD-1 expression similar to that in DS cells. It was observed that the transfected cells showed a similar inhibition in syncytialisation and hCG secretion as DS trophoblasts did. They also found that levels of hPL, hCGα, hCGβ and placental growth hormone

but not leptin mRNA were significantly lower in the SOD-1 transfected cells. This was similar to the reduction in the concentration of these proteins observed in DS placentae. Because transfection of cells with SOD-1 expressing vector was not entirely effective, the second study tagged SOD-1 with green fluorescent protein (GFP) to allow visualisation of the transfected protein in the cultured trophoblast cells. They noted that the cells that showed positive GFP-SOD-1 reactivity did not syncytialise while cells in the same culture dish that did not express the GFP-SOD-1 fused to form syncytium. This study suggested that the defects in syncytialisation observed in DS placental cells may well be attributed, at least in part, to the over expression of SOD-1 (Evain-Brion et al. 2000; Frendo et al. 2001).

1.5 Factors Affecting hCG Production & Release

In vitro studies have revealed many factors that alter the rate of synthesis and release of hCG from the placenta. Among these factors are cytokines such as inhibin and activin, and growth factors such as epidermal growth factor (EGF) and transforming growth factor beta 1 ($TGF\beta_1$). The control mechanisms, in which these factors are involved, are complex and highly inter-related.

Inhibin has been shown to inhibit hCG release from placental cytrophoblasts as they syncytialise (Petraglia, 1997), while activin stimulates its production (Steele et al. 1993; Song et al. 1996; Petraglia, 1997). Conversely, hCG can act to increase the production of inhibin and decrease the production of activin, thereby acting in a self-regulatory manner. It remains unclear whether these factors affect hCG production or release directly, or act via gonadotrophin releasing hormone (GnRH). $TGF\beta_1$ has been found to suppress hCG secretion from placental cells, as well as suppress placental cell differentiation (Morrish et al. 1991a). $TGF\beta_1$ may act synergistically with activin-A to suppress inhibin-A secretion from placental cells, however $TGF\beta_1$ alone shows no affect on inhibin-A secretion and activin-A alone shows only a slight reduction in inhibin-A release. Activin-A may also inhibit hCG induced inhibin-A production. It has also been observed that EGF acts synergistically with testosterone to significantly increase inhibin-A release (Qu and Thomas, 1993) (fig 1.5).

1.5.1 Inhibins and Activins

Both inhibins and activins are members of the transforming growth factor beta (TGF-β) superfamily which includes many hormones, growth factors and cytokines (Kingsley, 1994; Knight, 1996a). Their physiological functions are diverse, including regulation of cell growth, proliferation, adhesion, apoptosis and immunoregulation (Vale et al. 1988). All members of the family are structurally related, and most exert their actions at type I and type II serine/threonine kinase receptors.

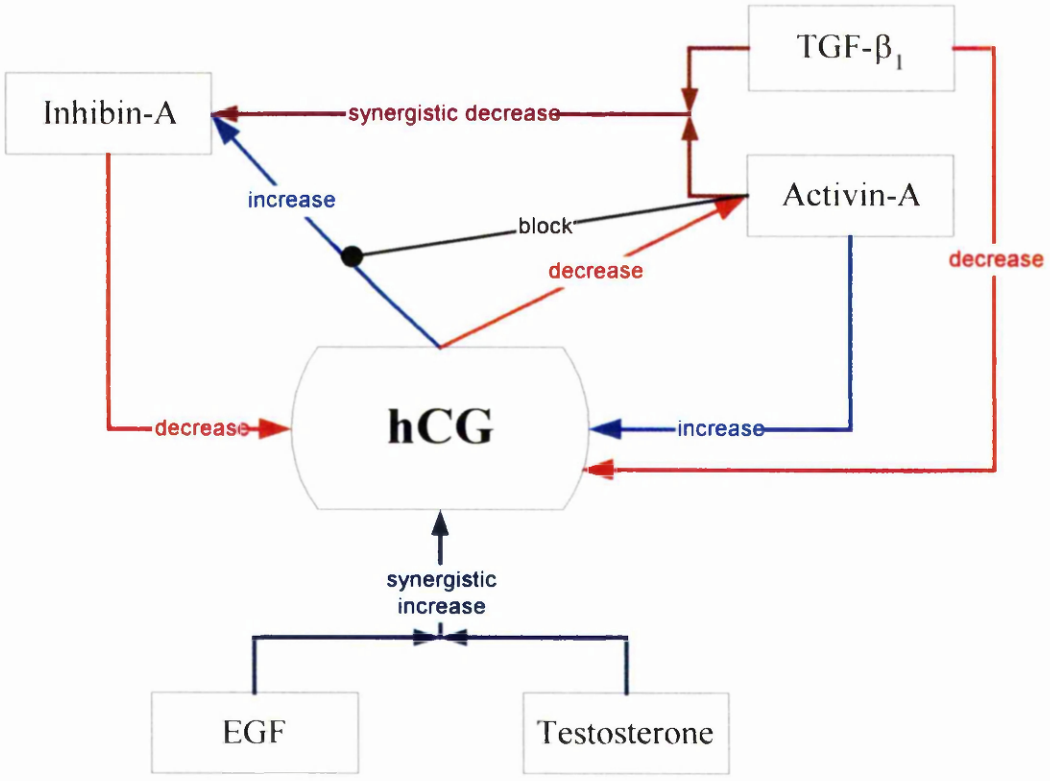


Figure 1.5 *Summary of the inter-relationships between hCG, EGF, TGFβ1, activin-A, inhibin-A and testosterone as shown by in vitro cell culture systems. The words "increase" and "decrease" are used to indicate where one factor increases or decreases either the production or release of another factor from placental cells.*

1.5.1.1 Structure and Localisation

Inhibin

Mature inhibin is a 31-32 kDa heterodimeric glycoprotein composed of an alpha and either a beta A (β_A), beta B (β_B) or beta C (β_C) subunit, which are linked by disulphide bonds. These arise from longer precursor molecules, which undergo post-translational modification to form the mature subunits. Inhibin-A has the structure $\alpha\beta_A$, while inhibin-B is of the form $\alpha\beta_B$, and inhibin-C has the structure $\alpha\beta_C$ (Robertson et al.

1985; Vale et al. 1986). Free subunit forms of inhibin can also be found in different tissues (fig 1.5.1.1). Inhibin was first discovered in male and female gonads, but was later shown to be present in many tissues throughout the body (Bilezikjian and Vale, 1992). In women, the main biologically active form of inhibin is inhibin-A. During early pregnancy the corpus luteum secretes inhibin-A, however the principal source of inhibin-A during pregnancy is thought to be the placenta. *In vitro* studies have localised inhibin-A production and secretion in the placenta to trophoblast cells, which were shown to store inhibin in small vesicles within the cytoplasm prior to secretion (Qu et al. 1992).

Activin

Activin is a 24kDa homodimer of the beta subunits, which like the alpha and beta subunits of inhibin, are linked by disulphide bonds. Activin may be one of three different forms: activin-A, which has the structure $\beta_A\beta_A$, activin-B which is comprised of two β_B subunits, or activin-AB which is of the form $\beta_A\beta_B$ (fig 1.5.1.1). Activins containing the β_C subunit have not been isolated, despite the theoretical fact that the β_C subunit is able to bind to the other β subunit forms. The most predominant form of circulating activin in females during both the normal menstrual cycle and pregnancy was found to be activin-A. The primary source of activin-A during pregnancy is thought to be the placenta (Petraglia et al. 1987; Petraglia et al. 1993; Petraglia et al. 1994a). Activin-B was found to be essentially absent from maternal serum, however it was found to be abundant in the AF and fetal cord serum prior to parturition (Petraglia et al. 1993).

1.5.1.2 Subunit Localisation

Several studies have looked at the localisation of the subunits of inhibin and activin by immunohistochemistry (Petraglia et al. 1991; Petraglia et al. 1992; Minami et al. 1992; McCluggage et al. 1998). These studies have, however, given slightly different localisations for the α and β_A subunits. The study by Petraglia *et al* (1992) found that both the α and β_A subunits could be localised to all trophoblastic cells, while an earlier study by the same group (Petraglia et al. 1991) found that the β_A subunits were widely distributed but the α subunits were confined to the cytotrophoblast cells. In contrast, two different studies found the α subunits to be confined to the syncytial cells (Minami et al. 1992; McCluggage et al. 1998), with one of these studies concluding that β_A

subunits were also found only in the syncytial layer (Minami et al. 1992). The reason for the variations in these results could be the different sources of the antibodies used in the immunohistochemical localisation experiments of the different groups. Only McCluggage *et al* (1998) used monoclonal antibodies that were proven to be highly specific for the different inhibin subunits (Groome et al. 1990; Groome and Lawrence, 1991). A UK company (Serotec) also manufactures immunoassays for the detection of inhibins and activins in biological fluids using these new antibodies (section 1.5.1.4).

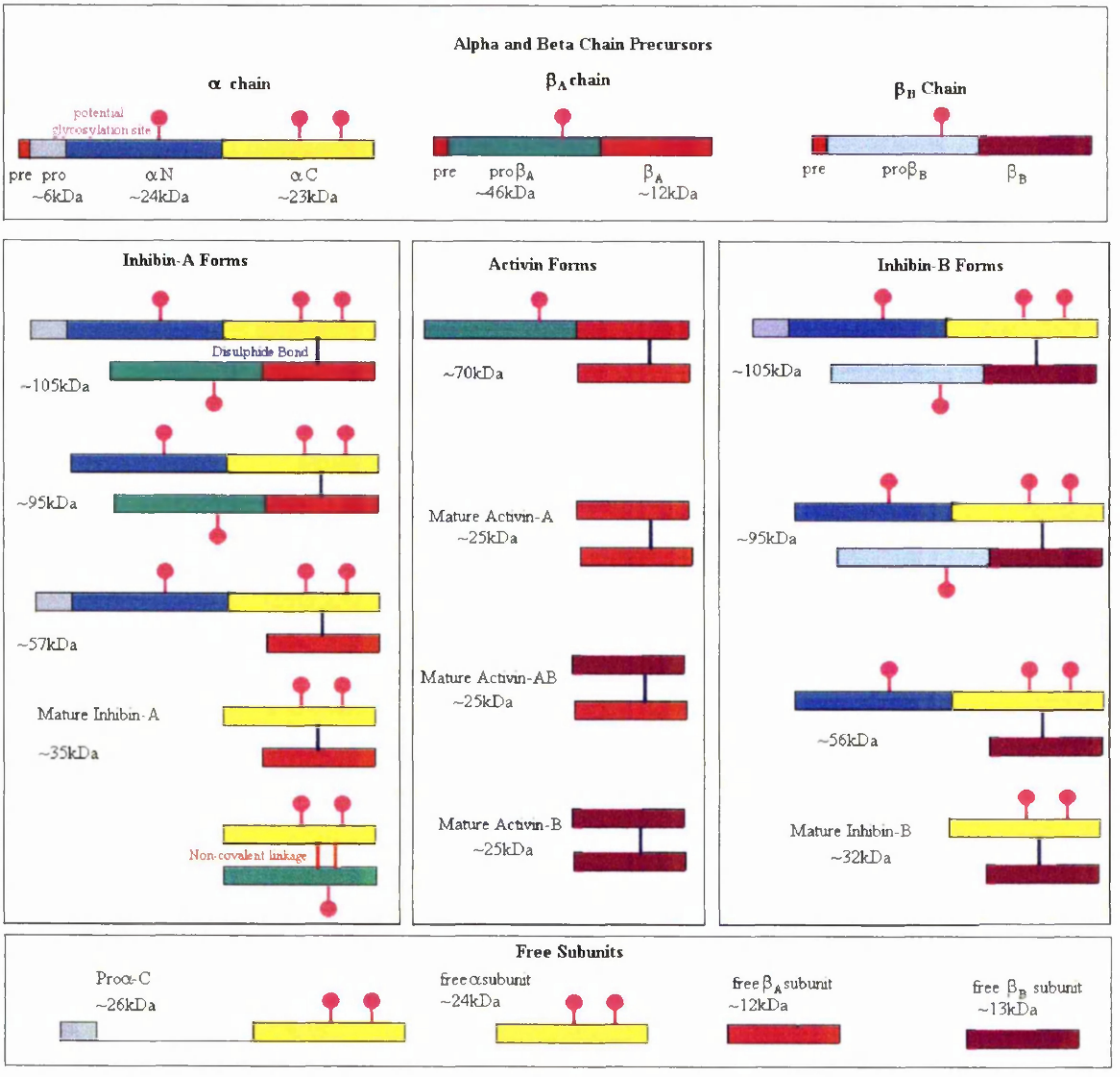


Figure 1.5.1.1 *Structure and approximate molecular weights of the forms of inhibin and activin and free subunits isolated in humans.*

1.5.1.3 Activin and Inhibin Binding Proteins

Inhibin and activin have been found complexed with the 725kDa protein alpha 2 macroglobulin (α2M). This binding protein does not seem to alter the biological activity

of either inhibin or activin. It is known that α2M circulates in the blood at levels in excess of those observed for activin and inhibin, thereby providing ample opportunity for the formation of α2M complexes. The biological relevance of the formation of complexes with α2M remains unclear, however it is thought it may play a role in the clearance or delivery of inhibin and activin (Vaughan and Vale, 1993; Krummen et al. 1993). In contrast to α2M, the 30-40kDa binding protein called follistatin renders activin biologically and immunologically inactive. Follistatin binds to one of the β subunits with approximately equal affinity to the binding of activin with its receptor. It has been suggested, therefore, that follistatin plays an important role in limiting the activities of activin on target cells. Follistatin has also been shown to bind to the inhibin β subunit, although the effect of this binding on the activity of inhibin has yet to be elucidated (Shimonaka et al. 1991; Nakamura et al. 1991; Krummen et al. 1993; Schneyer et al. 1994; Mathews, 1994; Petraglia et al. 1994b). The serum level of follistatin has been shown to be elevated during pregnancy when compared with the non-pregnant state. The follistatin level continues to rise throughout gestation, until parturition, although this elevation does not match the degree of elevation in activin levels observed towards the end of pregnancy (Wakatsuki et al. 1996).

1.5.1.4 Assaying Inhibin and Activin

The dimer structure of both activin and inhibin can complicate the matter of assaying the concentration of specific isoforms of inhibin and activin in biological fluids or tissues. Some assays were found to cross react with either different isoforms of the glycoprotein being measured, or with free subunits such as proαC. An example of such an assay is the Monash radioimmunoassay (McLachlan et al. 1986), which was unable to distinguish between dimeric inhibin and the free alpha subunit (Robertson et al. 1989; Schneyer et al. 1990). Newer assays have now been developed that are specific for the dimeric forms of inhibins and activins (Groome, 1991; Groome and O'Brien, 1993)

The inhibin/activin binding proteins α2M and follistatin can also cause interference in assays by masking the epitopes recognised by the immunoassay. These complications mean that early experiments using older, less specific assays often show conflicting results to those carried out more recently using the more sensitive assays (Groome and O'Brien, 1993; Groome et al. 1994; Muttukrishna et al. 1996; Knight et al. 1996b).

1.5.1.5 *Physiological Actions of Inhibin and Activin*

Activins and inhibins have many important physiological functions including roles in the control of embryogenesis, osteogenesis, haematopoiesis and reproduction. In the female reproductive system, both inhibin and activin are involved in the control of the menstrual cycle and pregnancy. Ovarian inhibin has a negative feedback mechanism on the production of FSH by the anterior pituitary, with inhibin suppressing FSH secretion, and FSH increasing the production of inhibin. Activin has also been shown to alter the secretion of pituitary hormones such as LH, adrenocorticotrophic hormone (ACTH), growth hormone (GH) and FSH. Activin can act antagonistically to inhibin, increasing the secretion of FSH from the pituitary (Muttukrishna and Knight, 1991). *In vitro* studies have shown that inhibin, activin and follistatin also have local autocrine/paracrine actions on the follicle, oocytes, corpus luteum and ovary. They have been shown by several studies to modulate hormone production, cell differentiation and maturation in these tissues (Knight, 1996a).

1.5.1.6 *Inhibins and Activins During Pregnancy*

The levels of both inhibin-A and activin-A are greatly elevated in the serum of pregnant women when compared with the levels observed in the serum of non-pregnant individuals (Qu et al. 1991; Tovanabutra et al. 1993; Muttukrishna et al. 1995; Muttukrishna et al. 1996). One function of elevated circulating inhibin-A may be to suppress FSH secretion from the pituitary, which would augment the actions of estradiol and progesterone to prevent follicular development during pregnancy. Although the corpus luteum produces significant quantities of inhibin during pregnancy, it is thought that the placenta is the major source of inhibin and activin, particularly towards the latter stages of pregnancy. *In vitro* studies have shown that activin can induce GnRH induced hCG release, while inhibin suppresses hCG secretion from placental cells (Qu and Thomas, 1993; Steele et al. 1993; Song et al. 1996).

Like inhibin-A and activin-A, the level of circulating follistatin is elevated in the serum of pregnant women, when compared with non-pregnant individuals. In the final trimester of pregnancy, the circulating levels of activin-A increase such that they exceed the levels of follistatin. Consequently, free activin-A is available to act in an endocrine manner at target cells (Woodruff et al. 1997). It is likely that there are still many

autocrine/paracrine roles for activin, follistatin and inhibin in the fetoplacental unit, which are yet to be elucidated.

1.5.1.7 Inhibin-A and Activin-A in DS Pregnancies

Inhibin-A

As mentioned in section 1.3.4 maternal serum levels of inhibin-A are significantly elevated in DS pregnancies, and it has been suggested that this marker could improve the detection rate of DS pregnancies achieved using the current hCG, AFP screening strategy. In contrast, AF levels of inhibin-A are significantly lower in DS pregnancies. The placental levels of inhibin and the inhibin subunit mRNA have been studied in DS placentae (Debiève et al. 2001). This study found no significant change in placental levels of inhibin-A or inhibin pro αC although there was a trend towards increased levels in the DS samples. The median inhibin-A level was 1.29MoM and inhibin pro αC level was 2.66MoM. Inhibin subunit mRNA levels were not significantly altered in DS placental samples. This study was based on only 6 DS samples, which were collected from 15 to 32 weeks gestation. A different study looked at placental inhibin subunit mRNA levels in 6 DS placentae and found significantly elevated α subunit mRNA in DS placentae. No significant difference in β_A subunit mRNA levels was found although there was a trend towards a slight reduction (Lambert-Messerlian et al. 1998).

Activin-A

There have been fewer studies carried out on the differences in activin-A levels between DS and unaffected pregnancies. Lambert-Messerlian *et al.* have carried out three studies each looking at activin-A levels in the maternal serum of DS and control pregnancies. In 1996, they reported a small but statistically insignificant increase in the DS levels of activin-A to 1.16 MoM using an immunoassay that detected only free activin (Lambert-Messerlian et al. 1996a). Later that year the same group repeated this study when a different assay became available which could measure both free activin-A, and activin-A that had been bound follistatin, (Lambert-Messerlian et al. 1996b). Again they reported a slight increase in activin-A levels from affected pregnancies (to 1.25 MoM), but on this occasion the increase was statistically significant. The latest study carried out by this group has reported a small, but statistically insignificant decrease in the maternal serum levels of activin-A from DS pregnancies (Lambert-Messerlian et al. 1998). In this latest study, the mRNA levels of activin/inhibin subunits in placental tissues were examined. In accordance with the activin-A results, β_A subunit mRNA showed slightly

lower levels of expression in DS placental tissues, however this difference was not statistically significant. Debiève et al (2001) noted a non-significant increase in placental activin-A levels in DS placentae to 1.45MoM. As mentioned before this study found no difference in the activin-A subunit mRNA in DS placentae. Activin-A and follistatin levels have been studied in maternal serum from DS pregnancies with a view to determining their usefulness as maternal serum screening markers (Cuckle et al. 1999). Activin-A levels were significantly elevated to 1.19MoM in the 30 DS cases studied but follistatin levels were not significantly altered (0.93MoM). Despite finding a significant elevation in activin-A levels, it was concluded that due to the large overlap in control and DS levels, this would not make a useful screening marker.

1.5.2 Transforming Growth Factor Beta (TGF-β)

1.5.2.1 Structure and Localisation

Transforming growth factor beta (TGF-β) is a member of a larger family of factors – the transforming growth factor beta superfamily. TGF-β is a polypeptide growth factor which can be released by most cell types throughout the human body, including the reproductive organs and placenta (Dungy et al. 1991). In humans, three subtypes of TGF-β have been isolated: TGF-β_1, TGF-β_2, and TGF-β_3. All are synthesised as large precursor proteins (pre-pro TGF-β), which contain an N-terminal signal peptide, a pro-segment known as latency associated peptide (LAP) and the C-terminal polypeptide, which is the mature TGF-β monomer. The active form of TGF-β has a dimeric structure usually composed of two identical disulphide linked subunits, although heterodimers of TGF-β_1 and TGF-β_2 subunits have been isolated. It is thought that LAP prevents the interaction of the mature TGF-β with receptors thereby rendering the factor inactive (fig 1.5.2.2) (Derynck and Choy, 1998). *In vivo*, activation of TGF-β is thought to be mediated by proteolytic cleavage of the pro form to yield the bioactive factor. *In vitro*, TGF-β can be processed to the bioactive form by acid, alkali or heat treatment (Cui and Ackhurst, 1996). TGF-β has many other associated binding proteins, which inhibit the activities of the growth factor. One example is latent TGF-β binding protein (LTBP), which binds to LAP by a disulphide bond. Another of these proteins is α2M, which also forms complexes with inhibin and activin, but unlike the situation with inhibin and activin, α2M sequesters the activity of TGF-β. Two secreted proteoglycans, namely decorin and biglycan also bind TGF-β with high affinity (Derynck and Choy, 1998).

Decorin has been localised to similar locations in the placenta as TGF-β (Lysiak et al. 1995). Several collagens, thrombospondin and fibronectin also bind TGF-β with high affinity, which illustrates that the availability of bioactive TGF-β is controlled at many levels. It is thought that TGF-β is only activated when it is in close proximity to the membrane receptors it complexes with.

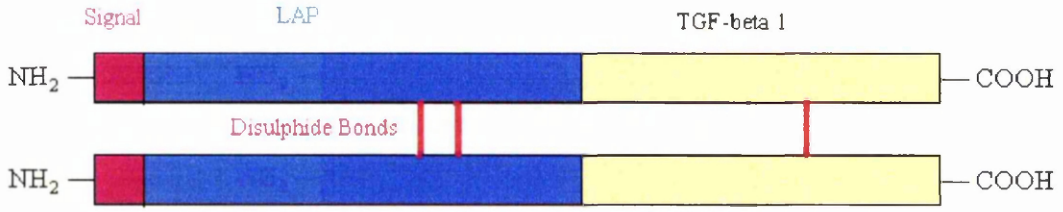


Figure 1.5.2.1 *Diagram of pre-pro TGF-β_1 dimer including the signal peptide, LAP and the mature TGF-β_1 segments.*

TGF-β is present in several ovarian tissues during both the normal menstrual cycle, and pregnancy (Chegini and Williams, 1992). TGF-$\beta_{1,2+3}$ mRNA expression has been noted in villous and decidual tissues throughout gestation (Dungy et al. 1991; Lysiak et al. 1995; Ando et al. 1998; Caniggia et al. 1999; Schilling and Yeh, 2000; Simpson et al. 2001). This mRNA expression has been primarily localised to the syncytiotrophoblast, decidual and stromal cells with variable reports on cytotrophoblast reactivity (Lysiak et al. 1995; Ando et al. 1998; Caniggia et al. 1999). Protein Immunolocalisation, largely carried out on paraffin wax embedded tissues, largely followed the reports on mRNA localisation. The most widely studied isotypes are TGF-β_{1+2} often using an antibody that reacts with both subtypes although more recent studies employ isotype specific antibodies. TGF-$\beta_{1,2+3}$ have principally been localised to the villous syncytium throughout gestation (Dungy et al. 1991; Minami et al. 1992; Chegini and Williams, 1992; Graham et al. 1992; Vuckovic et al. 1992; Lysiak et al. 1995; Caniggia et al. 1999; Schilling and Yeh, 2000) although two studies by the same group, which unlike the other studies used unfixed frozen tissue, found no syncytial staining in either first or third trimester placentae (Simpson et al. 2001; Lyall et al. 2001). In contrast villous cytotrophoblast cells have generally shown no TGFβ reactivity (Vuckovic et al. 1992; Lysiak et al. 1995; Simpson et al. 2001; Lyall et al. 2001) although one study found TGF-$\beta_{1/2}$ staining in cytotrophoblast cells (Ando et al. 1998) and one reported

cytotrophoblast TGF-β_3 staining (Caniggia et al. 1999). TGF$\beta_{1,2+3}$ have been localised to EVT (Lysiak et al. 1995; Schilling and Yeh, 2000; Simpson et al. 2001; Lyall et al. 2001) although the reports by Lyall *et al* (2001) failed to find any TGFβ_1 immunostaining. The decidua has been noted as a site of intense TGFβ_{1+2} reactivity particularly during early pregnancy (Graham et al. 1992; Lysiak et al. 1995; Ando et al. 1998; Simpson et al. 2001; Lyall et al. 2001). At term Lyall *et al* (2001) found no decidual TGF-β_1 expression. One study noted *in vitro* secretion of TGFβ_1 from a number of decidual cell types, first trimester cytotrophoblast and the JEG-3 choriocarcinoma cell line (Jokhi et al. 1997). TGF-β type I and II receptors have been localised to EVT, syncytium, cytotrophoblast, decidua and myometrium indicating an endocrine/paracrine role for the TGF-β family in placental development (Hatthachote et al. 1998; Ando et al. 1998; Schilling and Yeh, 2000).

1.5.2.2 Physiological Actions of TGF-β

Depending on the tissue and subtype, TGF-β seems able to both stimulate and suppress proliferation of cells. The ability of TGF-β_1 to suppress cellular proliferation has been of particular interest as a tumour suppressor drug. Its *in vivo* performance was, however, discovered to be less favourable than had been observed *in vitro* (Robinson and Rose, 1992). TGF-β has been implicated in many biological functions including embryogenesis, reproductive function, growth and development, inflammation and repair, as well as host immunity (Shull and Doetschman, 1994; Clark and Coker, 1998). TGF-β_2 has been shown to inhibit the *in vitro* proliferation of first trimester and term placental trophoblasts, (Graham et al. 1992). A study using cultured human trophoblasts has shown that exogenous TGF-β_1 has an inhibitory action on the production of hCG and aromatase activity (estrogen production) (Morrish et al. 1991a; Song et al. 1996). The reason for this inhibition remains unclear. It could either be due to a direct inhibition in the production of these hormones, or it could be because TGF-β_1 inhibits the differentiation of cytotrophoblast cells to syncytiotrophoblasts, an occurrence which has been associated with the increased production of hCG (Morrish et al. 1991a). TGF-β_1 and activin-A synergistically inhibited inhibin secretion from cultured human placental cells, however TGF-β_1 alone showed no effect on inhibin-A secretion (Qu and Thomas, 1993).

1.5.2.3 TGF-β Levels in Pregnancy

One study looking at the changing expression of TGF-β_1 in the placenta noted peak expression at around seventeen weeks with a second smaller peak observed in the latter stages of pregnancy at around 34 weeks (Dungy et al. 1991). Caniggia *et* al (1999) reported a peak in TGFβ_3 expression at eight weeks of gestation but could find no alteration in either TGF-β_{1+2} expression during this period. No gestational differences in expression or protein concentration of any of the TGF-β isoforms with gestation were noted in first trimester and term studies (Simpson et al. 2001; Lyall et al. 2001). The levels of TGF-β type I and type II receptor expression was shown to be elevated in the human myometrium during pregnancy, when compared with the non-pregnant levels. The level of receptor expression in the myometrium drops at parturition, while the concentration of TGF-β_1 remains elevated when compared to the non pregnant state (Hatthachote et al. 1998). Lawton *et al* (1997) isolated a novel form of TGF-β expressed in the placenta, which they named placental transforming growth factor-β (PTGFB). Although detectable in other adult tissues, PTGFB was expressed at much higher concentrations in the placenta.

1.5.2.4 TGF-β Levels in Abnormal Pregnancy

Few studies have been carried out on TGFβ_1 levels in abnormal pregnancies. One study has noted that maternal plasma levels of bioactive TGFβ_1 were significantly higher in pre-eclampsia when compared with normotensive pregnancies (Djurovic et al. 1997). In contrast no difference was found in the second trimester AF concentration of TGF-β_1 in pregnancies that subsequently went on to develop preeclampsia (Heikkinen et al. 2001). No difference was reported in placental expression of any of the TGF-β isoforms, studied by immunohistochemistry and ELISA and RT-PCR analysis of placental extracts, in either first trimester or term pregnancies complicated with preeclampsia (Simpson et al. 2001; Lyall et al. 2001). In contrast Caniggia *et al* (1999) reported a significant elevation in placental TGF-β_3 in third trimester preeclamptic placentae. Significantly higher plasma bioactive TGFβ_1 has been noted in pregnant women suffering miscarriage and non-pregnant women with a history of recurrent miscarriage (Ogasawara et al. 2000). DS AF levels of total TGFβ measured by bioassay were found to be significantly elevated when compared to normal pregnancy (Bromage et al. 2000).

1.5.3 Serine Threonine Kinase Receptors

Activin and TGF-β have been shown to exert their actions via serine/threonine kinase receptors. Activation of these receptors is achieved when the ligand forms a heteromeric complex of the type I and II receptors. In humans, there are two different type II activin receptors known as activin receptor type II (ActRII) and activin receptor type II_B ($ActRII_B$), both of which are present in human placental plasma membranes, as well as many other tissues in humans (Peng et al. 1993; Petraglia et al. 1994a). There are two forms of the type I receptor, known as ActRI and $ActRI_B$, both of which are unable to bind activin directly but either can form a heteromeric complex with the ActRII and activin, or the $ActRII_B$ and activin (Attisano et al. 1993; Matsuzaki et al. 1993; ten Dijke et al. 1993). Only one type I and one type II TGF-β receptor has been isolated in humans, known as TβR-I and TβR-II. The type II receptors are constitutively phosphorylated, while the type I receptors are phosphorylated only upon formation of the type I / type II receptor ligand complex (Mathews and Vale, 1993; Wrana et al. 1994). Receptor phosphorylation triggers a signal cascade, initiated by phosphorylation of the intracellular signalling molecule Smad2. This associates with Smad4 and the complex moves to the nucleus. Target genes are induced by binding of the Smad complexes directly to the promoter or by the Smad complex associating with other DNA binding proteins like FAST-1 (fig 1.5.3). Type III TGFβ receptors have been isolated, and are thought to act as ligand presenting receptors, however an equivalent activin receptor has yet to be discovered (McCarthy and Bicknell, 1993).

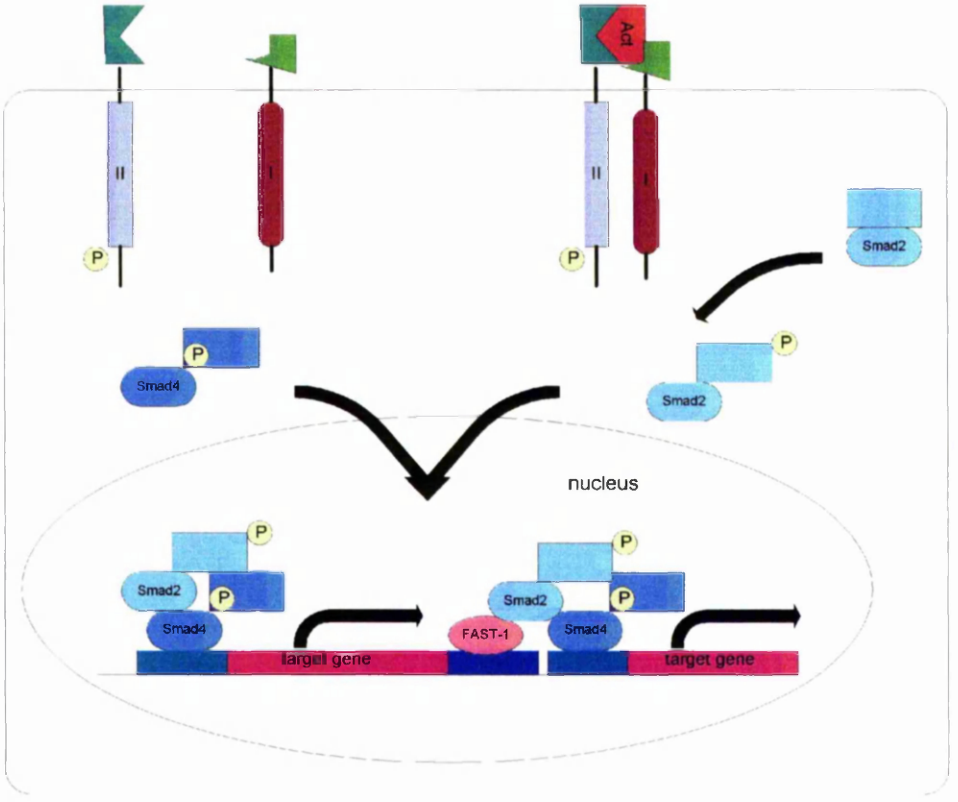


Figure 1.5.3 *Representation of activin receptor activation and signal transduction from the receptor to the nucleus. Upon ligand binding, the type I and II receptors associate triggering the phosphorylation of Smad2. Smad2 then joins with Smad4, entering the nucleus where they trigger target gene transcription either alone or in association with other DNA binding proteins like FAST-1.*

The search for the inhibin receptor has proven difficult and little is known about inhibin receptors. It was first discovered that despite having an affinity for activin receptors, inhibin also bound to cell surface receptors separate from activin receptors. (Woodruff et al. 1992). Further studies isolated a 120kDa protein from bovine pituitary extracts, which bound inhibin A. The peptide sequence was elucidated and the protein was named p120. A matching human cDNA sequence was found to match the p120 peptide sequence and expression of p120 mRNA was noted in rat pituitary and liver, and human and rat testis. Immunolocalisation of p120 in the rat testis indicated that it was primarily localised to Leydig cells, which are known to be a major target cell of inhibin and have been shown to bind inhibin on their surface (Chong et al. 2000). To date p120 is the most likely candidate to be an inhibin receptor.

1.5.4 Epidermal Growth Factor (EGF)

1.5.4.1 Structure and Localisation

Epidermal growth factor (EGF) is a 6.4kDa globular protein. It is synthesised as a longer pre-pro protein from which the mature form of EGF is released by proteolytic cleavage. The precursor consists of an N-terminal signal peptide, a pro-segment containing several EGF like repeats and a sequence, which shows homology to the LDL receptor (fig 1.5.4.1). The pro-segment also has a transmembrane hydrophobic domain, which suggests that the pro form of EGF may be membrane associated. The sequence of EGF is highly conserved between species, and shows homology to other factors like transforming growth factor alpha (Fisher and Lakashmanan, 1990).

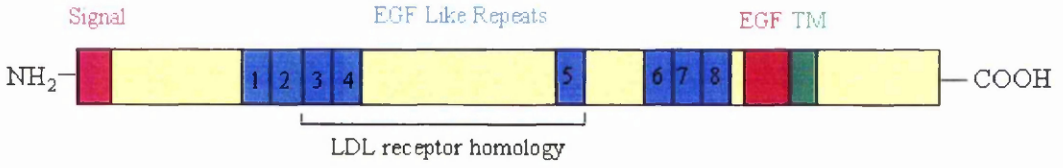


Figure 1.5.4.1 *Diagram of pre-pro EGF including the signal peptide, EGF like repeats, the area of LDL receptor homology and the hydrophobic transmembrane (TM) region.*

EGF can be found in most body fluids, for example urine, plasma, milk and saliva. Cells in many organs can synthesise this factor including the brain, kidney, stomach and salivary gland (Fisher and Lakashmanan, 1990). Both EGF and its receptors have been localised to cytotrophoblast cells and syncytium of the placenta (Ladines-Llave et al. 1991; Maruo et al. 1995).

1.5.4.2 Physiological Actions of EGF

EGF is involved in many functions during embryonic development including promoting the development of teeth and lungs. In adults, it inhibits the secretion of gastric acids and controls hormone secretion from tumours. It also has effects on the central nervous system. EGF in part mediates wound healing along with other factors. EGF stimulates the proliferation of many types of epidermal and epithelial cells and also embryonic cells. The proliferation of some cells is however inhibited by EGF and EGF can also induce the differentiation of some cell types (Fisher and Lakashmanan, 1990). Studies on the effects of EGF on term trophoblast cell cultures have shown that it promotes syncytialisation and hCG and hPL secretion from these cells (Morrish et al. 1987). hCG

and hPL secretion from first trimester explants is also stimulated by EGF (Barnea et al. 1990; Maruo et al. 1995). EGF also acts as an anti-apoptotic factor in trophoblast cultures (Ho et al. 1999). It has also been shown to increase invasion of trophoblast cells in first trimester explants and isolated cytotrophoblast cells (Barnea et al. 1990; Bass et al. 1994; Maruo et al. 1995).

1.5.4.3 EGF Receptors

EGF receptors (EGFR) are expressed on the cell surface of most tissues. It is a 170kDa glycoprotein with intracellular tyrosine kinase activity, which may also be activated by TGF-α. Binding of ligand to the receptor causes activation of the kinase activity, which initiates a cellular signalling cascade (fig 1.5.4.3). EGF binds to the extracellular domain of the EGFR, forming an EGF-EGFR dimer. This induces two receptors to come together forming a dimer complex. ATP then binds the cytoplasmic kinase domain of either of the two EGF-EGFR dimers in the complex. The ATP-bound dimer complex interacts with another (EGF-EGFR) dimer complex, phosphorylating one of the four EGFR cytoplasmic tails on tyrosine 1068. This phosphorylation causes the binding of Grb2-Sos1, which is the first step of the EGF signalling cascade (Carpenter, 2000; Bogdan and Klämbt, 2001; Gschwind et al. 2001).

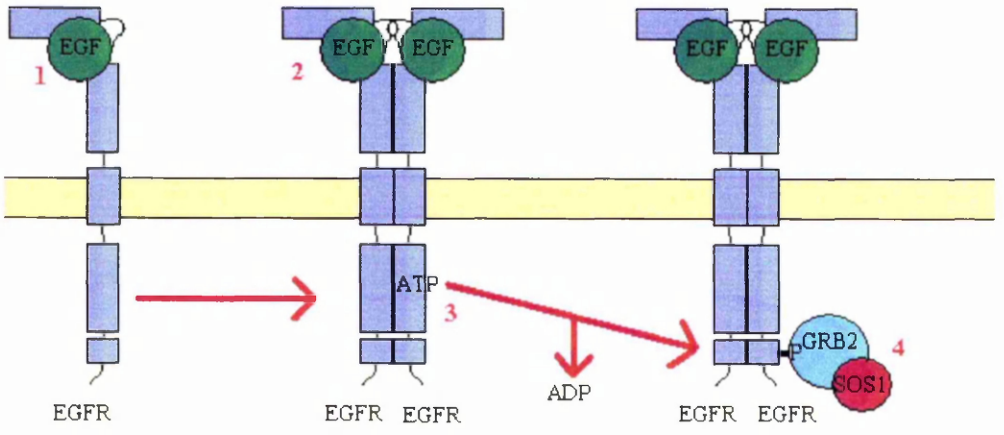


Figure 1.5.4.3 *Representation of the activation of the EGFR upon EGF binding.* ***1.*** *EGF binds to the EGFR.* ***2.*** *Two EGF bound receptors form and EGF-EGFR dimer complex.* ***3.*** *One of the kinase domains in the dimer complex binds ATP and phosphorylates tyrosine 1068 on the cytoplasmic domain of the EGFR.* ***4.*** *Phosphorylation of the tyrosine causes binding of the Grb2-Sos1 complex.*

1.5.4.4 EGF in Pregnancy

Maternal urine concentrations of EGF are slightly higher in the second trimester of pregnancy than levels in non-pregnant urine, though urinary levels in early pregnancy and at term are not significantly different from the non-pregnant concentration. The concentration increases between 6 and 20 weeks of gestation, and then declines towards the non-pregnant level at term (Hofmann et al. 1988; Watanabe, 1990; Moharam et al. 1992). In contrast, AF levels of EGF are higher towards term, correlating with gestational age (Watanabe, 1990; Varner et al. 1996). No correlation between maternal urine and AF levels of EGF can be observed indicating different sources of EGF in each compartment (Hofmann and Abramowicz, 1990; Moharam et al. 1992). Labouring women have the highest plasma levels of EGF and a function for EGF in the release of prostaglandins involved in the induction of labour has been suggested (Hofmann et al. 1988; Watanabe, 1990). EGFR have also been localised to cytotrophoblast cells and syncytium of the placenta indicating a role for EGF in placental development. EGF has been indicated as a stimulator of endometrial stromal cell proliferation (Ladines-Llave et al. 1991; Hofmann et al. 1991; Amemiya et al. 1994).

1.5.4.5 EGF Levels in Abnormal Pregnancy

Few studies have been carried out on the concentrations of EGF in abnormal pregnancies. Low maternal urine and AF EGF levels have been noted in pregnancies affected by intrauterine growth retardation (IUGR) (Hofmann et al. 1988; Watanabe, 1990; Varner et al. 1996; Lindqvist et al. 1999). One study failed to find this reduction in AF or urinary levels of EGF in women delivering small for gestational age babies (Moharam et al. 1992). EGF levels have not previously been studied in DS pregnancies.

1.6 Chromosome 21: Potential Factors Responsible for Maternal Serum Marker Changes in DS

1.6.1 DS Critical Region

The majority of the transcribed genes on chromosome 21 are situated on the long arm (q). Many studies have proposed that a region within the area 21q22 when present in triplicate is the minimum required to produce the DS phenotype. This has been termed the "DS critical region". There have, however, been many different reported "critical" regions (Shapiro, 1999) which suggests that it would be more appropriate to consider all

of the transcribed genes on chromosome 21 when trying to elucidate the mechanisms behind the phenotypic alterations observed in DS. Figure 1.6.1 illustrates the position of some genes of interest, which may be relevant to the biochemical and endocrinological changes noted in DS pregnancies.

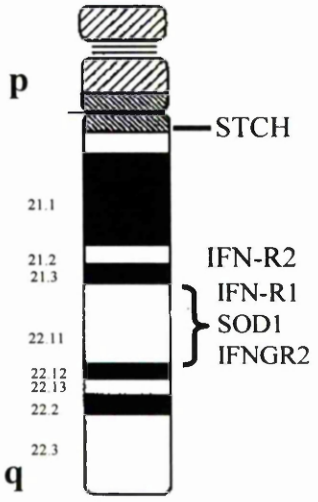


Figure 1.6.1

Diagram of Chromosome 21 indicating the relative positions of some genes of interest.

STCH=Stress 70 protein chaperone (21q11), IFN-R2=Type 2 interferon receptor (21q22.11), IFN-R1=Type 1 interferon receptor (21q22.11), SOD1=Superoxide dismutase (21q22.11), IFNGR2=Interferon gamma receptor 2 (21q22.11).

"Down's Syndrome Critical Region" lies within 21q22.

1.6.2 Interferon α/β Receptor (IFN-R1 & IFN-R2)

1.6.2.1 Receptor Structure

IFN α/β receptors are constitutively expressed on the surface of both IFN responsive and IFN insensitive cells. The interferon α/β receptor is composed of two subunits, the type 1 receptor (110kDa) and type 2 receptor (95-100kDa). Both subunits are required for cellular response to interferon (De Maeyer and De Maeyer-Guignard, 1998). The IFN-R2 is the ligand-binding component which, after IFN binding, complexes with the IFN-R1 (fig 1.6.2.1). Formation of the IFN-R2, IFN, IFN-R1 ternary complex then triggers an intracellular signalling cascade, namely the Jak/Stat pathway, which initiates the biological response to IFN stimulation of the cell (De Maeyer and De Maeyer-Guignard, 1998). Increased receptor expression has been noted in cells from DS patients as would be expected for a gene localised to chromosome 21 (Gerdes et al. 1993). The effects of this overexpression in placental development in DS pregnancies have not been studied.

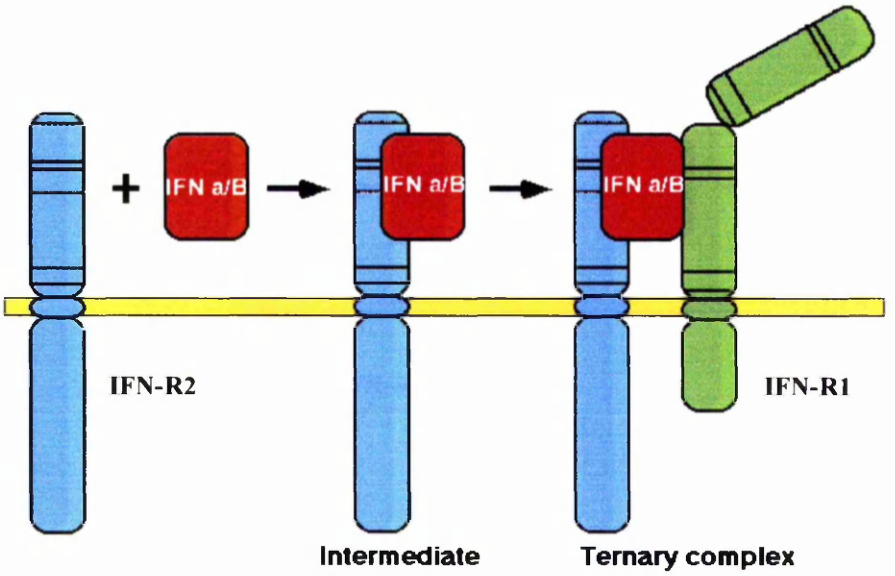


Figure 1.6.2.1 *Interaction of IFNα or IFNβ with its receptor. IFN first forms an intermediate complex with IFN-R2, the ligand-binding subunit of the receptor. This complex then binds the IFN-R1 to form a ternary complex.*
Modified from` "The Structure of Cytokine Receptors and their Signalling"
Rubstein, M et al [http://bioinfo.weizmann.ac.il/].

1.6.2.2 Jak/Stat Signalling Pathway

The binding of interferon to its receptor initiates the Jak/Stat signalling cascade. Jak is an abbreviation for Janus kinase, which is a family of protein tyrosine kinases, and Stat is an acronym for Signal Transducer and Activator of Transcription (Darnell, 1997). The first event in this pathway is activation of the kinases Jak1 and Tyk2 by phosphorylation. These kinases then phosphorylate either Stat1 (91, 84kDa) or Stat2 (113kDa) respectively on tyrosine residues causing the formation of Stat1-Stat2 heterodimers or Stat1-Stat1 homodimers. The heterodimer moves to the nucleus where it complexes with the DNA binding protein p48. This complex then binds the interferon stimulated response element (ISRE), which is found in the promoter region of various IFN stimulated genes, initiating gene transcription. The Stat1 homodimer can bind directly to the IFNγ activation site (GAS) initiating gene transcription (Darnell et al. 1994; Darnell, 1997; De Maeyer and De Maeyer-Guignard, 1998). Figure 1.6.2.2 summarises this pathway.

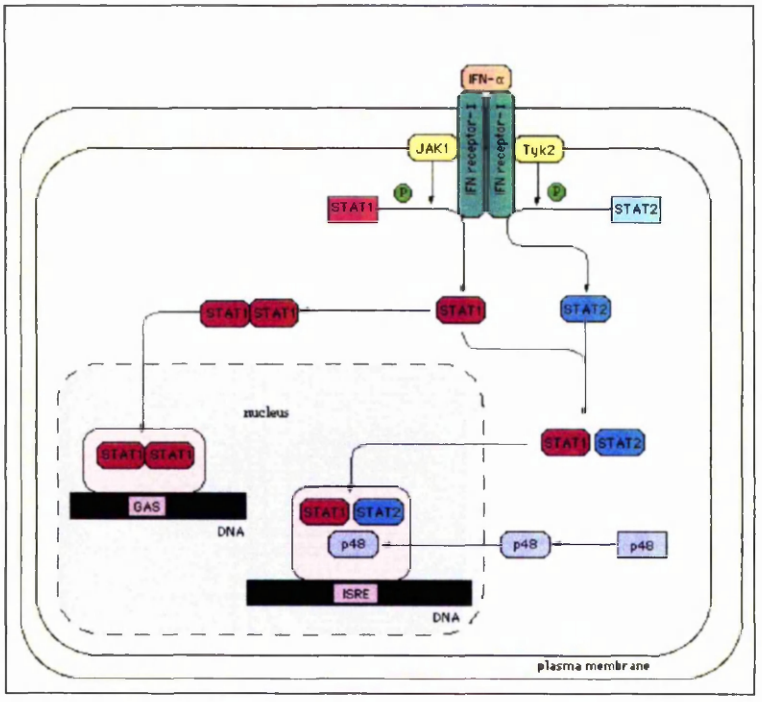


Figure 1.6.2.2

Summary of the Jak/Stat intracellular signalling cascade initiated by the binding of IFNα to cell surface receptors.

Modified from [http://www.grt.kyushu-u.ac.jp/spad/pathway/ifn_alpha.html].

1.6.2.3 Biological Responses to IFN α & β

IFNα

There are many different variants of IFNα, which have masses of 19-26kDa, all coded for by genes on chromosome 9. Monocytes, macrophages, lymphoblasts, fibroblasts and other cells produce IFNα following stimulation by viruses, hormones or nucleic acids. The main biological activities of IFNα are its potent antiviral actions and inhibition of the growth of some tumours. It has been shown to alter cytokine expression in some cell types (De Maeyer and De Maeyer-Guignard, 1998). IFNα and its receptors have been localised to the human placenta (Howatson et al. 1988; Paulesu et al. 1991; Paulesu et al. 1997; Bennett et al. 1999), and the role of IFNα in human reproductive physiology has been studied (Chard et al. 1986; Chard, 1989; Chard, 1991b). IFNα also inhibits hCG secretion from hCG secreting bladder carcinoma cells (Iles and Chard, 1989). Increased dosage of IFN-R1 & IFN-R2 in DS placentae may therefore have a role in the biological changes noted in maternal serum of these pregnancies.

IFNβ

IFNβ is a 20kDa glycoprotein coded for by genes on chromosome 9. It is synthesised mainly by fibroblasts and epithelial cells, and the production can be induced by viruses, double stranded RNA and invading pathogens. Some cytokines, for example tumour necrosis factor and interleukin 1 also induce its production. IFNβ plays an important

role in non-specific anti-viral responses. It stimulates the activity of NK-cells and the expression of HLA class I antigens, while blocking the expression of HLA class II antigens. The serum levels of beta-2-microglobulin and the synthesis of low affinity IgE receptors are also enhanced by IFNβ. Like IFNα, it inhibits the growth of some cells derived from solid tumours (De Maeyer and De Maeyer-Guignard, 1998).

1.6.3 Interferon Gamma Receptor 2 (IFNGR2)

1.6.3.1 Receptor Structure

In order for IFNγ to exert a biological response, the heterodimeric IFNGR1 (gene on chromosome 6) and IFNGR2 (gene on chromosome 21) must both be present on the cell surface (Soh et al. 1994; Hemmi et al. 1994). INFγ binds the receptor as a dimer, with each subunit of the dimer associating with one IFNGR1 subunit and one IFNGR2 (fig 1.6.3.1). As with the IFN-R1 and IFN-R2, this initiates a Jak/Stat signalling cascade (De Maeyer and De Maeyer-Guignard, 1998).

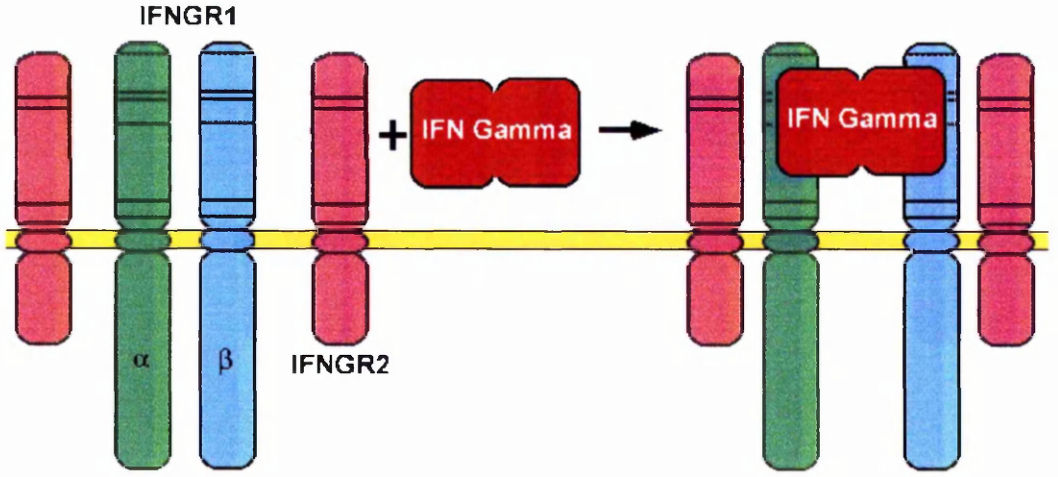


Figure 1.6.3.1 *Interaction of IFNγ with its receptor. IFNγ binds as a dimer the IFNGR1, which consists of an α and a β subunit. An accessory factor (IFNGR2) is required for this binding to occur.*
Modified from "The Structure of Cytokine Receptors and their Signalling"
Rubstein, M et al [http://bioinfo.weizmann.ac.il/].

1.6.3.2 Jak/Stat Signalling Pathway

The binding of IFNγ to its receptor initiates the Jak/Stat signalling cascade. The first event in this pathway is activation of the kinases Jak1 and Jak2 by phosphorylation. The activated kinases then phosphorylate Stat1α (91kDa) inducing the formation of Stat1α

homodimers. The homodimers translocate to the nucleus where they can bind directly to the GAS response element initiating transcription of IFNγ responsive genes (Darnell et al. 1994; Darnell, 1997; De Maeyer and De Maeyer-Guignard, 1998). Figure 1.6.3.2 summarises this pathway.

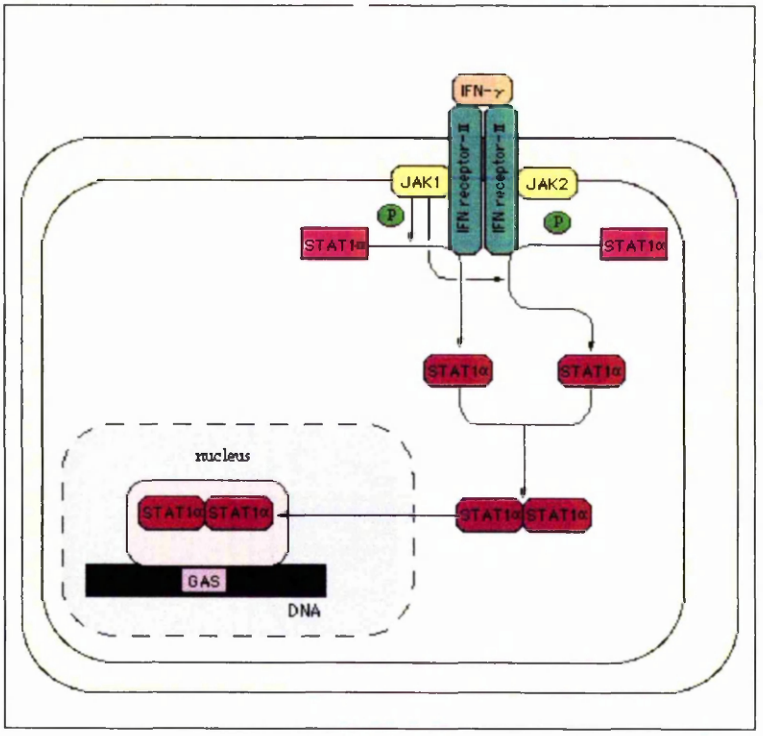


Figure 1.6.3.2

Summary of the Jak/Stat intracellular signalling cascade initiated by the binding of IFNγ to cell surface receptors.

Modified from [http://www.grt.kyushu-u.ac.jp/spad/pathway/ifn_gamma.html].

1.6.3.3 Biological Responses to IFN γ

IFNγ is a dimeric protein with each subunit having a molecular mass of 20 or 25kDa. The gene for IFNγ maps to chromosome 12. It is synthesised mainly by T-cells and NK-cells when induced by antigens or mitogens. It is also produced by lymphocytes that express CD4 and CD8 antigens. B-cells can also produce IFNγ. IFNγ has both antiviral and antiparasitic actions. It influences cell-mediated cytotoxicity and T-cell growth and also controls the expression of MHC class 2 genes. The anti-proliferative activities of IFNγ on many cell types, are the most pronounced of the IFN family (De Maeyer and De Maeyer-Guignard, 1998). IFNγ was shown by one study to inhibit hCG secretion from term trophoblast cultures without increasing the incidence of cell necrosis (Marth et al. 1989). This and another study (Yanushpolsky et al. 1993) investigated the effect of IFNγ administration to JAR cells. One study found no significant effects of IFNγ on

JAR cell function (Marth et al. 1989) while the other reported inhibition of growth and protein production but no differences in hCG release (Yanushpolsky et al. 1993).

1.6.4 Stress 70 Protein Chaperone (STCH)

Stress 70 Protein Chaperone (STCH) is a 60kDa microsomal associated protein. It is a member of the heat shock protein 70 (HSP 70) family and is constitutively expressed in all tissues (Ulrich, 1996). Although located outside the "Down's syndrome critical region" of chromosome 21, it has been show that expression is increased in tissues from DS patients (Tassone et al. 1999; Yoo et al. 1999). The sequence of the HSP 70 family of genes is highly conserved among species. The main functions of this family of proteins are to assist folding, assembly or disassembly of proteins; transport of proteins around the cell; degradation of unstable proteins and protein complexes; and to assist in the refolding of incorrectly folded proteins. One of the HSP70 proteins binds to hydrophobic amino acids of unfolded proteins thereby preventing them from forming inappropriate structures either within themselves or with other proteins. This binding event triggers ATPase activity in HSP 70 and the resulting energy is thought to be used to fold the protein (Ulrich, 1996).

1.7 Summary and Aims

1.7.1 Summary

As outlined above, there are many biochemical changes associated with DS pregnancies. Of particular interest are the altered maternal serum concentrations of hCG, AFP, UE_3 and inhibin-A because of their use as serum screening markers of DS pregnancies. The pathophysiological basis of these alterations has received limited study over recent years, often yielding conflicting results (sections 1.4.4 and 1.4.5.4). Further understanding of factors influencing the production and secretion of these markers could provide an insight into placental function in DS pregnancies. Advances in knowledge of placental function and standardisation of culture methods for *in vitro* analysis of placental trophoblast cells provide a platform for the investigation of biochemical alterations in DS pregnancies and the effects of increased expression of genes on chromosome 21. The aims of this study encompass these areas of interest.

1.7.2 Aims

1.7.2.1 Inhibin-A and Activin-A

- To compare the concentration of inhibin-A in maternal serum and placentae from control and DS pregnancies, to discover the basis of the elevation in the maternal serum concentration of this protein.
- To compare the concentration of activin-A in maternal serum and placentae from control and DS pregnancies to determine if there was any difference in the production or secretion of this protein in DS pregnancies.
- To correlate the inhibin-A and activin-A results with previously reported hCG concentrations in the same tissues.
- To localise the subunits of inhibin-A and activin-A in placental sections using immunohistochemistry to compare the localisation and relative staining intensities of the subunits between control and DS placentae.

1.7.2.2 Growth Factors Associated with Placental Function

- To compare the concentrations of EGF and $TGF\beta_1$ in maternal serum, placentae, AF and maternal urine from DS and control pregnancies to gain an insight into placental/foetal growth in DS.
- To correlate these results with hCG concentrations previously measured in these pregnancies.

1.7.2.3 Measurement of hCG mRNA Isolated from Placental Tissues

- To assess the sensitivity and practicality of northern blot analysis.
- To develop and validate an ELISA based method of mRNA quantification.
- To compare the ELISA method to a real time PCR method of mRNA quantification.

1.7.2.4 Placental Trophoblast Culture

- To optimise a standardised protocol for placental trophoblast isolation in a department where this work had not previously been carried out.
- To fully characterise the purity of isolated cells and monitor their behaviour in culture using standardised protocols.
- To study the effects of cryopreservation on trophoblast viability.

1.7.2.5 *Effects of IFNα on Placental hCG Production*

- To localise IFN-R1 and IFNGR2 in control and DS placentae using immunohistochemistry and compare staining intensity between both groups.
- To analyse the potential effect of IFN-R1 over expression on hCG secretion from DS placentae by analysing the effect of IFNα administration on hCG secretion from normal term trophoblast cultures.
- To determine effect of IFNα administration on hCG secretion and hCG mRNA production by term placental trophoblast cultures.

Section 2

Materials and Methods

2.1 Sample Collection

2.1.1 Placental Samples

Pieces of placenta that were previously snap frozen in liquid nitrogen were retrieved from storage at -70°C, and thawed. Control samples were collected between 1992 and 1996 following termination of pregnancy (TOP) for psychosocial reasons, and for the Down's syndrome (DS) cases between 1992 and 1998 following therapeutic TOP. TOP was by induction of labour using Mifepristone/RU486. Use of placental tissue was approved by the Yorkhill NHS trust ethics committee. Details of the gestations as determined from the date of last menstrual period, or if the dates were uncertain from ultrasound scan, are outlined in table 2.1.1.

Gestation (weeks)	Control Samples	Down's Samples
14	9	3
15	3	1
16	11	3
17	7	9
18	5	9
19	6	13
20	2	12
21	0	4
22	0	4
23	0	2
Total	**43**	**60**

Table 2.1.1 *Number of control and DS placental samples available at each gestation.*

The cytogenetic profile of all DS foetuses was determined from either amniotic fluid samples or chorionic villus samples. Four of the women were referred for cytogenetic profiling following an abnormal ultrasound scan, three were referred because of advanced maternal age, with the remainder detected by routine AFP/hCG biochemical screening.

Placental extracts were prepared as previously described (Newby et al. 1997). A cross section of tissue of approximately 0.2g was washed in phosphate buffered saline (0.01M phosphate buffer, 0.0027M potassium chloride and 0.137M sodium chloride, pH 7.4, PBS), blotted on absorbent tissue, and homogenised in a Potter-Elvehjem homogeniser with 1ml of extraction buffer (0.1% Triton-X-100 in PBS). The homogenate was then transferred to a 1.5ml micro centrifuge tube and sonicated on ice (2 x fifteen seconds at 4μm). The resulting homogenate was spun for fifteen minutes at 10,000g, and the supernatant transferred into a clean micro centrifuge tube. The placental extracts were stored at -40°C until the marker assays were performed.

2.1.2 Whole Placentae for Cell Isolation

Normal term placentae were collected within 30min of delivery by caesarean section. In all cases, informed parental consent was obtained following approval of the use of placental tissue by the Yorkhill NHS Trust ethics committee.

2.1.3 Maternal Serum Samples

Clotted blood samples were collected from pregnant women having routine second trimester screening for DS and neural tube defects, and the serum separated by centrifugation. Half was used for the routine hCG/AFP screening, with the remainder stored at -20°C. Serum samples that were collected and stored by the Institute of Medical Genetics between 1992 and 1998 were retrieved from frozen storage and used in this study. The gestational range of the serum samples used in this study is outlined in table 2.1.3.

2.1.4 Amniotic Fluid Samples

Amniotic fluid samples from which large cellular material had been removed by centrifugation for foetal chromosome analysis, were retrieved from frozen storage. The DS amniotic fluid samples were collected from the same cases from which placental samples were obtained. A random selection of 53 samples from unaffected pregnancies, matched for gestation and time of collection with the DS cases were used as a control group. The gestational range of all amniotic fluid samples is summarised in table 2.1.4.

Gestation (weeks)	Control Samples	Down's Samples
15	20	21
16	20	22
17	10	9
18	10	2
19	10	0
20	10	1
Total	**80**	**55**

Table 2.1.3 *Number of control and DS maternal serum samples available at each gestation.*

Gestation (weeks)	Control Samples	Down's Samples
15	6	3
16	11	10
17	9	20
18	10	13
19	11	3
20	6	1
Total	**53**	**50**

Table 2.1.4 *Number of control and DS amniotic fluid samples available at each gestation.*

2.1.5 Maternal Urine Samples

Control and DS maternal urine samples, from first and second trimesters of pregnancy, were retrieved from frozen storage for use in this study. They were collected between 1994 and 2000 and spun to remove particulate material before storage at -20°C. Some of the second trimester DS samples also had matched serum and placental samples. Table 2.1.5 outlines the number of urine samples available at each gestation.

Gestation (weeks)	Control Samples	Down's Samples
7	0	1
8	0	1
9	0	0
10	10	1
11	10	4
12	10	7
13	10	9
14	10	0
15	10	2
16	9	3
17	7	6
18	10	9
19	10	6
20	6	3
21	1	2
22	0	2
Total	**103**	**56**

Table 2.1.5 *Number of control and DS maternal urine samples available at each gestation.*

2.2 Cell Culture Methods

2.2.1 Cytotrophoblast Cell Isolation

The following buffer was prepared using Milli-Q RG ultrapure water and sterilised by filtering through a 0.22μm bottle top filter (Millipore). Culture medium was prepared using sterile supplies (Gibco BRL).

1x Hank's Balanced Salts Solution (HBSS) pH7.4 (Sigma)

5.36mM potassium chloride

0.44mM potassium phosphate monobasic

136.89mM sodium chloride

0.34mM sodium phosphate dibasic

5.55mM D-Glucose

4.17mM sodium bicarbonate

Supplemented Medium 199 (M199)

Medium 199

10% heat inactivated foetal bovine serum (FBS)

100U/ml penicillin G

100μg/ml streptomycin sulphate

0.25μg/ml amphotericin B

Placental trophoblast cell isolation was carried out using a method modified from that of Kliman *et al* (1986). The following method was scaled up or down accordingly if more or less tissue was used.

2.2.1.1 Placental Dissection

A placenta was obtained as soon as possible following caesarean section. In a lamina flow cabinet, using sterile instruments, the placenta was cut into cubes (approx. 4cm) avoiding the edges, the point of cord attachment or calcified areas, and placed into warm, sterile 0.9% saline. The cubes were washed 3-5 times in fresh warm saline to remove excess blood, leaving the tissue in the final wash of saline. The cubes were removed one at a time from the saline and healthy villous tissue dissected and placed in fresh saline until 30-35g tissue was collected. The villous tissue was drained through gauze and crudely minced before being washed again through gauze to remove traces of blood.

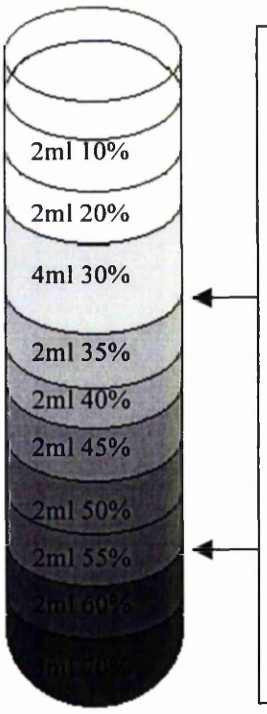
2.2.1.2 Enzymatic Digestion

The tissue was transferred to a 1l conical flask to which 150ml sterile HBSS was added followed by 15ml sterile 2.5% trypsin solution in Ca^{2+}/Mg^{2+} free PBS (Roche

Diagnostics Ltd.) and 30mg type IV deoxyribonuclease I (DNase) from bovine pancreas (Sigma). The flask was covered and incubated at 37°C, shaking gently in an orbital shaking incubator, for 30min. Following incubation, the flask was placed horizontally in the flow cabinet to allow the tissue to settle before approximately 100ml of supernatant was removed to a clean sterile beaker. A further 100ml HBSS, 10ml trypsin and 20mg DNase was added to the tissues and the flask returned to the shaking incubator for a further 30min. During this incubation period, the 100ml of supernatant that was removed from the flask was layered in 25ml aliquots on top of 5ml newborn calf serum (NCS, Gibco BRL) in 30ml tubes and spun at 1,000g for 10min. The supernatant was aspirated from the tubes and the cell pellet suspended in M199 and stored refrigerated until all cells could be pooled. Following the 30min incubation period, the placental tissue was allowed to settle as before and the supernatant removed and treated as described. Initially, a third digestion step was carried out however this was found to cause the villous tissue to break up leading to contamination of the trophoblast cells with villous fragments. All cells were pooled and spun at 1,000*g* for 10min to pellet them before suspending in 6ml M199.

2.2.1.3 Trophoblast Enrichment by Density Gradient Centrifugation

Two discontinuous percoll (Pharmacia) gradients were prepared by layering the stock concentrations into two 30ml glass corex tubes as described in figure 2.2.1.3 Suspended cells were layered on top of the percoll gradients (3ml per gradient) and were spun at 1,600*g* for 30min with the centrifuge brake switched off. Following centrifugation, several bands of cells were visible. The four bands between the marks on the tube (1.048-1.062g/ml), containing mononuclear cells were collected, pooled in a 50ml tube, topped up with M199 and spun at 1,000*g* to pellet the cells. The supernatant was poured from the cell pellet, which was re-suspended in 2ml M199 for counting. Cells were counted at a 1 in 100 dilution using 1 part M199, 1 part trypan blue solution (0.4% trypan blue in 0.81% sodium chloride and 0.06% potassium phosphate dibasic, Sigma) in an improved Neubauer haemocytometer. Cells were then seeded at the appropriate density (see table 2.2.3) or further purified by immunodepletion.



Percoll conc. (%)	Percoll (ml)	Sterile H_2O (ml)	10xHBSS (ml)
10	5	40	5
20	10	35	5
30	30	60	10
35	17.5	27.5	5
40	20	25	5
45	22.5	22.5	5
50	25	20	5
55	27.5	17.5	5
60	30	15	5
70	52.5	15	7.5

Figure 2.2.1.3 *Preparation of discontinuous percoll gradients. Table describes how each percoll solution was prepared using stock percoll, sterile water and 10x HBSS. Diagram illustrates the quantity of each percoll concentration layered to construct gradient. The tube was marked at arrows.*

2.2.2 Purification of Cytotrophoblast Cells by Immunodepletion

Contaminating cells were removed from isolated cytotrophoblasts using magnetic beads (Dynal UK Ltd.) coated with monoclonal mouse anti-HLA Class I antibody (Harlan Sera-Lab Ltd. UK). This has been shown to be an efficient way of purifying cytotrophoblasts because HLA Class I antigen is expressed on all contaminating cells but not trophoblasts (Kawata et al. 1984). Firstly, 100µl ($4x10^7$) Dynabeads Pan Mouse IgG were washed by suspending in 1ml of HBSS + 0.1% bovine serum albumin (BSA), then aspirating the wash buffer when the tube was in a magnetic holder. The beads were re-suspended in 100µl of wash buffer to which 4µl (2µg) anti-HLA antibody was added, and then incubated at 4°C on a roller mixer for approximately 1h to allow the beads to be coated with antibody. The beads were washed 3 times as before and re-suspended in

100µl of wash buffer. Cells were purified by addition of 50µl of coated beads for up to a maximum of $50x10^6$ cells recovered from percoll separation, in a total volume of 1ml. If more cells were purified, the number of beads was increased proportionally. The cell/bead suspension was incubated at 4°C on a roller mixer for 30 min to allow contaminating cells to bind to the beads. Following incubation, the now pure cells were removed from the beads, which were held in the tube using a magnetic stand, and plated at the appropriate density (table 2.2.3).

2.2.3 Culture of Isolated Cytotrophoblast Cells

Following isolation, cells were plated on culture treated dishes (Sterelin) at the densities outlined in table 2.2.3. Cells were cultured at 37°C, 5% CO_2, in M199. The cells were cultured for 24h to allow them to adhere to the culture surface, before the medium was removed and the cells washed using HBSS to remove any loose cells. The culture medium was replaced and subsequently changed at 24h intervals. Aspirated medium was stored at -40°C for further analysis.

Well Diameter	Culture Area	Wells/Plate	No. of Cells	Medium/Well
35mm	$9.5cm^2$	6	$4x10^6$	4ml
22.1mm	$3.8mm^2$	12	$2x10^6$	2ml
15.6mm	$1.9mm^2$	24	$1x10^6$	1ml

Table 2.2.3 *Trophoblast plating density in each size of culture dish, and the quantity of M199 used.*

To estimate plating efficiency, the number of cells that had failed to adhere to the culture surface after 24h was counted using an improved Neubauer haemocytometer. Culture medium was transferred directly to the counting chamber without further dilution. As a different method of estimation of cell number, cells were scraped into PBS/0.1% triton-X-100 (100ml per 10^6 cells) and stored at -40°C until a protein or DNA assay was carried out on the lysates.

2.2.4 Addition of Interferon to Trophoblast Cell Cultures

Cells were seeded on 12-well culture dishes at the appropriate density (table 2.2.3) and cultured in M199 for 24h to allow the cells to adhere to the culture surface. Culture medium was changed and replaced with M199 containing 1, 10 or 100U/ml IFN α (Serotec, UK). IFNα had previously been reconstituted in HBSS/0.1% BSA solution at a 100 times final concentration to allow 20μl of this solution to be added to the 2ml of M199 in each culture well. Some cells also received diluent only or no addition to act as controls. All additions were made to triplicate wells. At 24h periods, culture medium was collected and stored at -40°C, and replaced with new medium with the appropriate addition. At each time point, cells were pooled from the triplicate wells in tri-reagent (Sigma) and stored at -70°C until RNA, DNA and protein could be extracted (section 2.5.1).

2.2.5 Cell Freezing

Cells were cryopreserved according to the method described by Yui et al (1994). Immediately following isolation, cytotrophoblast cells were suspended (10^7 cells/ml) in FBS with 10% dimethyl sulphoxide (DMSO) and transferred to 1.8ml cryovials (Nunc). Tubes were wrapped in several layers of bubble wrap and placed in a -70°C freezer for 24h. The cells were then transferred to liquid nitrogen for long-term storage. Cells were recovered by thawing in a 37°C water bath, suspending in approximately 45ml M199 and centrifuging to pellet. The cells, now washed free of DMSO, were re-suspended in M199 at the appropriate density and seeded into culture dishes as outlined previously (section 2.2.3).

2.3 Immunocytochemistry & Immunohistochemistry

2.3.1 Preparation of frozen tissue sections

Pieces of placental tissue, previously collected and snap frozen in liquid nitrogen, were retrieved from storage at -70°C and sections (6μm) cut using a Leica cryostat at -20°C. Sections were adhered to superfrost plus slides (BDH) and allowed to air dry for 20min

before fixing in acetone (BDH) for 20min, followed by air-drying for a further 20min. Sections were stored sealed at -20°C until required.

2.3.2 Preparation of Cytospins

Wet fixed cytospins were prepared using a Shandon Cytospin® cytocentrifuge system. Freshly isolated trophoblast cell suspension was pipetted into a sample chamber ($1x10^5$ cells in 0.5ml PBS) to which 6 drops of CytoFixx™ cytological fixative (Cell Path, UK) were added. The cells were cytospun at 1500rpm for 3min to allow the cells to adhere to superfrost plus slides. After removal from the cytocentrifuge, a further 2 drops of CytoFixx™ were dropped onto the circle of cells on each slide. Cytospins were stored sealed at -20°C until required.

2.3.3 Fixation of Trophoblast cells in Culture Dishes

Trophoblast cells growing in culture were fixed to allow immunostaining of cells at various stages of differentiation. Culture medium was removed from the cells, which were then washed using PBS. Ice-cold methanol (BDH) was added to the culture dish, and placed in a -20°C freezer for 20min. Following fixation, the cells were washed 3 times using PBS, and were stored refrigerated in PBS until required.

2.3.4 Avidin/Biotin Immunoperoxidase Staining on Frozen Sections

Frozen tissue sections were allowed to return to room temperature, loaded into a Sequenza staining chamber (Shandon, UK) and washed using PBS. Non-specific staining was blocked by incubating the sections for 10min at room temperature in 100µl normal goat serum (SAPU, NGS) diluted 1:4 using PBS. Appropriately diluted primary antibody (see table 2.3) was added to each section (100µl) and incubated for 1h. The sections were washed for 5min using PBST (PBS + 0.3% Triton-X-100) before 100µl solution 1 (biotinylated goat anti-mouse and anti-rabbit secondary antibody in PBS) of the duet staining system (Dako Ltd., UK) was added to each slide and incubated at room temperature for 35min. The sections were washed as before, then 100µl of solution 2 (biotinylated horse radish peroxidase + avidin) of the duet system was added to each

section. This was incubated for 35min before being washed as before, then removed from the Sequenza and washed for 5min in water. Antibody binding was visualised by incubating for 2-10min in a diaminobenzidine (DAB), hydrogen peroxide solution (KEMENTECH, Denmark). Once the desired strength of colour was reached, sections were washed for 5min in water. Sections were counterstained in haematoxylin, differentiated in acid alcohol (0.25% conc. hydrochloric acid in methylated spirits) and rinsed in Scotts tap water (0.04M sodium bicarbonate, 0.16M magnesium sulphate in water) to intensify the blue staining. The sections were dehydrated through methylated spirits, 2 changes of ethanol, 2 changes of histoclear (National Diagnostics) and mounted using synthetic resin (Ralmount, BDH). Slides were viewed using an Olympus light microscope. Matched control and DS sections were stained in the same batch. Each batch included a negative control, either omitting the primary antibody or replacing the primary antibody with control mouse immunoglobulin fraction.

2.3.5 Avidin/Biotin Immunoperoxidase Staining on Fixed Cells in Culture

PBS was removed from the culture dish and non-specific staining was blocked by incubating the cells for 30min at room temperature in a covering of NGS (1:50 using PBS). The serum was poured off and enough of the appropriately diluted antibody (see table 2.3) was added to cover the culture surface. This was incubated at room temperature for 45min before being washed (3x5min) using PBS. The duet staining system was used as previously described, followed by visualisation using DAB. Dishes were filled with PBS and viewed using an Olympus CK2 inverted light microscope.

2.3.6 Fluorescent Immunohistochemistry & Immunocytochemistry on Frozen sections & Cytospins

Tissue sections or cytospins were allowed to return to room temperature before being loaded into the Sequenza staining system as described before. Non-specific staining was blocked by incubating the sections for 20min at room temperature with 100µl NGS (1:9 using PBS). Antibody was diluted (see table 2.3) in PBS + 0.1% BSA, added to the

slides and incubated at room temperature for 1h. Slides were washed for 15min using PBS + 0.1% Triton-X-100. Fluorescein isothiocyanate (FITC) conjugated goat anti-mouse secondary antibody (Dako Ltd., UK), diluted 1:20 in PBS/BSA, was added to each slide and incubated, shielded from light, for 1h. All subsequent steps were carried out in the dark. The slides were washed as before and mounted in fluorescent mounting medium (Dako Ltd., UK) containing 0.75μM propidium iodide. When complexed with DNA, propidium iodide absorbs light at 493nm, and fluoresces red (630nm). This allows nuclei to be visualised by microscopy. Slides were viewed using an Olympus fluorescence microscope and images were captured using an Olympus camera and IP Lab software.

2.3.7 Fluorescent Immunocytochemistry on Fixed Cells in Culture

The protocol outlined for avidin/biotin immunocytochemistry (section 2.3.5) was followed until the wash step following incubation with the primary antibody. FITC conjugated secondary antibody was added to the dishes and incubated at room temperature for 45min. The dishes were washed as described before, then drained. The cells were mounted with a round coverslip, using two drops of fluorescent mounting medium containing 0.75μM propidium iodide. Cells were visualised using the fluorescence microscope described previously.

Antigen	Clone	Supplier	Dilution
Inhibin α subunit	R1	Serotec Ltd. UK	1:50
Inhibin/activin β_A subunit	E4	Serotec Ltd. UK	1:75
CD 45	2B11 & PD7/26	Dako Ltd. UK	1:100
Vimentin	V9	Novo Castra Laboratories Ltd. UK	1:100
Cytokeratin 7	LP5K	Autogen Bioclear UK Ltd.	1:20
CD 9	P1/33/2	Dako Ltd. UK	1:20
CD163	Ber-MAC 3	Dako Ltd. UK	1:20
Fibroblast specific antigen (FSA)	ASO2	Oncogene Research Products, USA	1:20
HLA Class I	W6/32	Harlan Sera-Lab Ltd., UK.	1:20
Placental alkaline phosphatase (PLAP)	8B6	Autogen Bioclear UK Ltd.	1:50
Desmoplakin 1&2	DP2.15	Autogen Bioclear UK Ltd.	1:50
Interferon α/β receptor (rabbit polyclonal)	C18	Santa Cruz Biotechnology, UK	1:100
Interferon γ receptor β (rabbit polyclonal)	N20	Santa Cruz Biotechnology, UK	1:100
Cytokeratin 5/6/18	LP34	Novo Castra Laboratories Ltd. UK	1:20
Goat $F(ab')_2$ anti mouse immunoglobulin FITC conj.		Dako Ltd. UK	1:20

Table 2.3 *Suppliers of antibodies, and dilutions used in immunocytochemistry and immunohistochemistry. All antibodies are mouse monoclonal unless otherwise stated.*

2.3.8 Comparison of Immunohistochemistry Staining Intensities

The difference in relative staining intensities between gestation matched control and DS placental sections was compared using a semi-quantitative method. Two independent scorers assessed the staining intensity, blinded to the identity of the section, using the criteria outlined in table 2.3.8. One scorer gave two sets of independent scores for each section, while the other scorer gave one score for each section. The average score was then calculated for each section. In some cases different areas of the section were scored separately i.e. stroma and trophoblast layer scored separately.

Score	Degree of Staining
-	No staining
+/-	Light and patchy staining not on all villi
+	Light staining on the majority of villi
++	Medium to strong staining
+++	Strong to very strong staining

Table 2.3.8 Criteria for scoring the intensity of immunohistochemical staining of placental sections.

2.4 Assays

2.4.1 Protein Assay

The protein level of each placental sample was determined to standardise all further measurements against, using a method modified from that of Lowry *et al* (1951). This assay involves the pre-treatment of the samples with an alkaline copper solution to which a solution of sodium tungstate, molybdate and phosphate (Folin-Ciocalteau reagent) is added. The copper-amino acid complex (primarily involving tyrosine and tryptophan) reduces the Folin-Ciocalteau reagent producing a blue colour, which absorbs light at 690nm.

Prior to commencing the assay, three solutions were prepared: A (2% sodium carbonate in 0.1M sodium hydroxide), B_1 (1% copper sulphate in distilled water) and B_2 (2% sodium potassium tartrate in distilled water). Next, freeze dried BSA was reconstituted in distilled water to give a 500μg/ml stock solution. From this stock solution, dilutions were carried out to give a series of 7 standards with concentrations ranging from 0 to 500μg/ml. Before the commencement of each assay, a solution $A/B_1/B_2$ (98%/1%/1% respectively) was prepared. Placental samples were diluted either 1 in 5 or 1 in 10, using distilled water and protein isolated from cultured trophoblasts were diluted 1 in 10.

Firstly, 20μl of diluted sample or BSA standard was pipetted in duplicate into wells of a microtitre plate, to which 200μl of the prepared $A/B_1/B_2$ solution was added. The microtitre plate was shaken gently before covering and incubating at room temperature for 15 min. Following incubation, 20μl of Folin-Ciocalteau phenol reagent (BDH), diluted 1:1 with distilled water, was added to each well of the microtitre plate. This addition was made while the plate was shaking to ensure immediate, thorough mixing. The plate was incubated at room temperature, in the dark, for 45 min. Finally, the absorbance of each well was read at 690nm on a Labsystems Multiskan® Bichromatic plate reader, and the protein concentration of each sample determined from the BSA standard curve (using Genesis software).

2.4.2 Inhibin-A Immunoassay

The concentration of inhibin-A present in the placental homogenates was determined using a medium sensitivity inhibin-A dimer assay kit (Serotec Ltd., UK). This is a solid phase enzyme linked immunosorbent sandwich assay, which has a pre-treatment step to improve the specificity and affinity of the assay, and allow haemolysed samples to be assayed. The antibodies used in this assay make it highly specific for inhibin-A, and it therefore does not cross-react with other species of inhibin, or free alpha subunits (Groome, 1991; Groome and O'Brien, 1993).

The protocol supplied with the assay was followed for the previously prepared samples of placental homogenate, which were diluted (either 1 in 10 or 1 in 20) in the supplied foetal calf serum. The inhibin-A standards were also diluted using foetal calf serum giving a range of 0-2000pg/ml.

The basic steps of the protocol firstly involved 50µl of sodium dodecyl sulphate (SDS) being added to 100µl of sample or standard in micro centrifuge tubes, which were then capped and placed in a boiling water bath for 3min. After cooling, 100µl of assay diluent, and 50µl of 6% hydrogen peroxide were added to each sample, and incubated at room temperature for 30min. Following incubation, 80µl of treated sample was added, in duplicate to the wells of the antibody coated microtitre plate, which was then covered and incubated for 2h at room temperature. The plates were then washed 4 times using the supplied wash buffer and drained on absorbent paper before 50µl of the alkaline phosphatase conjugated anti inhibin-A α subunit antibody was added to each well. The plate was covered and incubated at room temperature for 30min. After incubation, the plate was thoroughly washed 8 times, as described above, leaving the final wash solution to soak in the wells for 15min. The plate was then washed a further 3 times, before draining the plate, and adding 50µl of substrate solution to each well. The plate was covered and incubated at room temperature for 30 min to allow the reaction to proceed. Each well of the plate then received 50µl of amplifier solution and was mixed by gentle agitation. The plate was covered and incubated at room temperature for approximately 40 min, until the absorbance of the 2000pg/ml standard at 490nm reached 2.0. Once the desired absorbance was achieved, 50µl of stop solution (0.46M sulphuric acid) was added to each well and the absorbance values for each well were determined at 490nm, referenced at 620nm. The standard curve was plotted using four-parameter logistic software (4-PL), and the values of the samples extrapolated from this.

2.4.3 Activin-A Immunoassay

The concentration of activin-A present in the placental homogenates was determined using an ultrasensitive activin-A dimer assay kit (Serotec Ltd., UK). This assay has similar properties to the inhibin-A assay, making it highly specific and allowing the total activin-A concentration of each sample to be determined (Groome, 1991; Groome and O'Brien, 1993). The protocol supplied with the assay was followed for the previously prepared samples of placental homogenate which were diluted (either 1 in 50 or 1 in 100) in the supplied 5% BSA in PBS. The supplied activin-A standards were also diluted using BSA in PBS giving a range of 0-5000pg/ml.

The basic steps of the protocol firstly involved the addition of 125µl of SDS to 125µl of sample or standard in micro centrifuge tubes, which were then capped and placed in a boiling water bath for 3 min. After cooling, 20µl of 30% hydrogen peroxide was added to each sample, and incubated at room temperature for 30min. Meanwhile, 25µl of assay diluent was pipetted into each well of the supplied antibody coated microtitre plate. Following incubation, 100µl of treated sample was added, in duplicate to the wells of the microtitre plate. To this 25µl of reconstituted biotinylated anti inhibin-A β_A subunit antibody was added, and the plate was covered and incubated at room temperature overnight on a plate shaker. On the following day, the plates were washed 4 times using the supplied wash buffer, allowing the buffer to soak in the wells for 15sec between each wash. The plate was allowed to drain on absorbent paper before 50µl of diluted streptavidin alkaline phosphatase solution was added to each well of the microtitre plate. The plate was then covered and incubated at room temperature for 1h. After incubation, the plate was thoroughly washed 8 times, as described before, leaving the final wash solution to soak in the wells for 15 min. The plate was then washed a further 3 times, by immersing the plate in fresh wash buffer. After draining the plate, and adding 50µl of substrate solution to each well, the plate was covered and incubated at room temperature for 2h to allow the reaction to proceed. Each well of the plate then received 50µl of amplifier solution and was mixed by gentle agitation. The plate was covered and incubated at room temperature for approximately 20 min, until the absorbance of the 5000pg/ml standard at 490nm reached 1.7 absorbance units. Once the desired absorbance was achieved, 50µl of stop solution was added to each well and the absorbance values for each well were determined at 490nm, referenced at 620nm. The standard curve was plotted using 4-PL software, and the values of the samples extrapolated from this.

2.4.4 Transforming Growth Factor Beta1 (TGFβ_1) Immunoassay

The TGF β_1 ELISA (Promega, UK) is specific for bioactive TGF β_1, showing less than 2% cross reactivity with either TGFβ_2 or TGFβ_3 (Promega data sheet). The following buffers were prepared for use in this assay using distilled deionised water:

DPBS (pH 7.35)

2.7mM potassium chloride
140mM sodium chloride
1.47mM potassium phosphate monobasic
8.1mM sodium phosphate dibasic
0.9mM calcium chloride 2 hydrate
0.49mM magnesium chloride 6 hydrate

Carbonate Coating buffer

25mM sodium carbonate
25mM sodium bicarbonate

TBST Wash Buffer

20mM Tris-HCl (pH 7.6)
150mM sodium chloride
0.05% v/v Tween 20

To allow measurement of total levels of this growth factor, samples were firstly acid pre-treated to process the latent form of TGFβ_1 to the bioactive form of the protein. Prior to acid treatment, serum placental and amniotic fluid samples were diluted 1 in 5 using DPBS. Samples were acidified by adding 10µl 1N hydrochloric acid to 100µl diluted sample, incubating for 15 min, and then neutralising by addition of 10µl 1N sodium hydroxide. Placental and serum samples were further diluted 1 in 20 (final dilution 1 in 100) using the supplied diluent, while amniotic fluid samples were not further diluted. All samples were assayed in duplicate following the protocol supplied with the kit.

Prior to the commencement of the assay, 96 well microtitre plates (Nunc MaxiSorp) were coated with monoclonal antibody by adding 100µl diluted antibody (10µl antibody in 10ml carbonate coating buffer) to each well and incubating overnight for 17h at 4°C. The antibody solution was flicked from the plate and 270µl of supplied block buffer (diluted 1 in 5 with water) was added to each well of the plate and incubated at 37°C for 35 min. This solution was removed from the wells, and the plate washed once using

TBST wash buffer. Each well of the coated plate received 100µl of prepared sample or standard (0-1000pg/ml), was sealed and then incubated at room temperature shaking for 90min. The samples were removed from the plate, which was washed 5 times using TBST. Anti TGFβ_1 polyclonal antibody (diluted 1:1,000) was added to each well of the plate (100µl per well) and incubated at room temperature shaking for 2h. The plate was washed 5 times before 100µl horseradish peroxidase (HRP) conjugated secondary antibody, diluted 1:2,000, was added to each well of the plate and incubated for 2h. During this incubation time, the enzyme substrate solution was prepared by adding 5ml tetramethylbenzidine (TMB) solution to 5ml peroxidase substrate, which was mixed and stored shielded from light until required. Following incubation, the plate was washed a final 5 times using TBST before addition of 100µl of prepared substrate solution. This was incubated at room temperature for 15 min to allow development of a blue colour. The reaction was stopped by addition of 100µl 1M phosphoric acid, which changed the colour to yellow. The absorbance was read at 450nm and the sample results calculated from a standard curve plotted using 4-PL software.

2.4.5 Epidermal Growth Factor (EGF) Immunoassay

The EGF assay (R&D Systems, UK) is a highly specific ELISA with a limit of detection of less than 0.7pg/ml. Amniotic fluid samples were assayed undiluted, and urine samples were assayed at a 1 in 300 or 500 dilution in supplied urine sample diluent, both according to the manufacturer's recommended protocol for urine samples. Serum samples were assayed at a 1 in 20 dilution in supplied serum sample diluent following the serum protocol. The standards (n=7) used to assay the serum and urine samples ranged from 3.9-250 pg/ml, while the amniotic fluid samples were assayed against a 7-point standard curve ranging from 0.97-62.5 pg/ml each constructed using the appropriate diluent. Each sample and standard was added in duplicate, 200µl per well, and the plate was sealed and incubated at room temperature for 2h. Samples were flicked from the plate, which was washed 3 times using the supplied wash buffer, before 200µl HRP conjugated anti EGF polyclonal antibody was added to each well of the plate. For the urine protocol the plate was incubated at room temperature for 1h while for the serum protocol this incubation was 2h. Following incubation, the plate was washed 3 times as before. To each well, 200µl substrate solution (TMB) was added and

incubated at room temperature for 20 min until a blue colour formed. The reaction was stopped by addition of 50µl of stop solution (2N sulphuric acid), which produced a yellow colour. The absorbance was read at 450nm corrected at 540nm and the values of the samples extrapolated from the standard curve which was plotted using 4-PL software.

2.4.6 Human Chorionic Gonadotrophin (hCG) Immunoradiometric Assay

The quantity of hCG secreted into culture medium by trophoblasts was measured using the MAIA clone™ magnetic solid phase immunoradiometric assay (BioChem ImmunoSystems UK Ltd.). Culture medium was assayed neat or diluted 1 in 2, 5 or 10 using fresh culture medium. The first step of the assay was addition of 25µl sample or standard (0-500mIU/ml) in duplicate, to 5ml plastic assay tubes, to which 250µl ^{125}I anti-hCG Reagent (^{125}I and fluorescein labelled anti-hCG monoclonal antibody solution) was added. The tubes were vortex mixed and incubated at 37°C for 15min. Next, 100µl Separation Reagent (sheep anti-fluorescein coated magnetic beads) was added to each tube and vortex mixed. The tubes were incubated at room temperature for 5min and then the rack of tubes were attached to a magnetic separator base and allowed to settle for 2min. While still in the separator, the supernatant was poured from the tubes, which were then inverted on paper towels and tapped to dislodge any drops of liquid in the tubes. The tubes each received 500µl of wash buffer, were vortex mixed and returned to the magnetic separator for 2min before the supernatant was removed as described before. The tubes and separator were left inverted on paper towels for 5 min to ensure removal of all drops of liquid. Each tube was counted for 2min in a Packard Cobra 5010 gamma counter. Log_{10} concentration of standards was plotted against counts per minute and a spline curve fit was carried out, from which the values of each sample were calculated. Positive and negative control samples were included in each assay.

2.4.7 Placental Alkaline Phosphatase (PLAP) Immunoassay

The concentration of PLAP released into the culture medium by trophoblasts was determined using the Innotest™ hPLAP immunoassay (Autogen Bioclear UK, Ltd.). Previously collected culture medium was assayed undiluted by adding 100µl of sample in duplicate to the wells of the supplied mouse monoclonal anti-hPLAP coated microplate strips, followed by 100µl assay buffer. Supplied standards (0-1000 mU/l) were also added in duplicate to the strips (200µl per well) before the plate was mixed, sealed and incubated for 3h at 37°C to allow the PLAP to bind to the immobilised antibody. The plate was washed 3 times using the supplied wash buffer and drained. Prepared substrate (P-nitrophenyl phosphate/ N-ethylaminoethanol solution) was added to each well (200µl) and mixed. The plate was incubated for 2h in the dark at 37°C to allow the immobilised PLAP to produce a yellow coloured product from the substrate. The absorbance of each well was measured at 405nm and the value of each sample calculated from the standards by linear regression. Controls supplied with the kit were included in each assay.

2.5 Western Blotting

The following buffers were prepared for use in western blotting:

Gel Buffer A (pH 8.8)
1.5M Tris
0.4% SDS

Gel Buffer B (pH 6.8)
0.5M Tris
0.4% SDS

RIPA Buffer
1% Igepal CA-630 (Sigma)
0.5% sodium deoxycholate
0.1% SDS
in PBS

PBST
2.5% Tween-20 in PBS

Running Buffer (pH 8.3)
125mM Tris
1M glycine
0.5% SDS

Loading Buffer (5x)

500mM Tris-HCl (pH 6.8)

50% glycerol

10% SDS

5% mercaptoethanol

0.5% bromophenol blue

Transfer Buffer

25mM Tris

0.2M glycine

0.5% SDS

in 20% methanol

2.5.1 Denaturing Gel Electrophoresis and Protein Transfer

A 12% resolving gel was prepared using 28.8ml 30% acrylamide (acrylamide:bis-acrylamide 37.5:1), 4.8ml 50% glycerol, 18ml buffer A, 20ml water, 360µl 10% ammonium persulphate (APS), 36µl TEMED. This was poured into the gel former and the air excluded using a layer of isopropanol. The stacker gel was prepared using 2.7ml 30% acrylamide, 5ml buffer B, 12.2ml water, 100µl 10% APS, 20µl TEMED. The isopropanol was washed from the resolving gel before the stacker gel was poured and combs carefully positioned to form the wells for sample loading. Samples that had previously been homogenised in RIPA buffer, 0.1mg/ml PMSF, aprotinin, 1mM sodium orthovanadate were diluted and mixed with the appropriate quantity of 2x loading buffer to allow addition of 20-100µg protein per lane. The samples were boiled for 5min then put on ice before loading into the gel. After the stacker gel was set, combs were removed and the gel transferred to the tank and top and bottom reservoirs were filled with running buffer. Samples and pre-stained SDS-page protein markers were loaded in the wells ensuring the same quantity of protein was added to each well. The gels were run at 15mAmps, 400volts for approximately 2h until separation of the marker bands was visible. Nitrocellulose (Hybond, Amersham Pharmacia) and filter paper were cut to the same size as the gel and soaked in transfer buffer. Sponges from the transfer equipment were also soaked in buffer. The stacker gel was trimmed from the top of the gel and the transfer sandwich was carefully assembled under transfer buffer. The protein was transferred to the membrane by electrotransfer (30volts, 250Amps) at 5°C, overnight.

2.5.2 Immunoblotting

The membrane was removed from the transfer apparatus and trimmed. Successful transfer of protein could be confirmed at this point by staining the membrane with 0.1% Ponceau S in 5% acetic acid (Sigma). This stains major protein bands red and is reversible by washing membrane in 0.1M sodium hydroxide for 1min. Non specific antibody binding was blocked by incubating the membrane in 5% powdered milk in PBST for 1h at room temperature. The primary antibody was diluted to the appropriate concentration (table 2.5.3) in 3% milk in PBST and incubated with the blot for 1h at room temperature. The membrane was washed twice for 5min in PBST before addition of secondary antibody (HRP labelled goat anti rabbit or goat anti-mouse, Amersham Pharmacia Biotech) at a 1:1000 dilution in PBST containing 3% powdered milk. This was incubated at room temperature for 1h before the membrane was washed three times for at least 10min each wash. The membrane was covered in a layer of luminol detection reagent (ECL western blotting detection reagent, Amersham Pharmacia Biotech) and incubated at room temperature for 1min. The blot was placed between plastic and exposed to film (Hyperfilm ECL, Amersham Pharmacia Biotech) in a light proof cassette containing intensifying screens and exposed at room temperature for up to 1h. Film was developed in a Fuji RGII x-ray film processor.

Antigen	Clone	Supplier	Diln.
IFN α/β receptor (rabbit polyclonal)	C18	Santa Cruz Biotechnology, UK	1:100
pSTAT1 (mouse monoclonal)	A-2	Santa Cruz Biotechnology, UK	1:100

Table 2.5.3 *Suppliers of antibodies, and dilutions used in western blotting.*

2.6 Molecular Methods

The following buffers were purchased for use during molecular methods:

MESA Buffer (pH 8.3) (Sigma)

40mM 3-(N-Morpholino) propanesulphonic acid (MOPS)

1mM Ethylenediaminetetra-acetic acid (EDTA)

10mM sodium acetate

DNA Loading Buffer (Promega, UK)

0.005% ethidium bromide

15% Ficoll 400

0.03% bromophenol blue

0.03% xylene cyanol FF

0.4% orange G

10mM Tris-HCL (pH7.5)

50mM EDTA

RNA Loading Buffer (Sigma)

62.5% deionised formamide

1.14M formaldehyde

1.25x MESA buffer

0.02% bromophenol blue

0.02% xylene cyanol

TAE Buffer (CP Labs)

40mM Tris-acetate (pH8.3)

1mM EDTA

TE Buffer (pH 8) (Sigma)

10mM Tris

1mM EDTA

20x SSC Buffer (pH 7) (Sigma)

0.3M sodium citrate

3M sodium chloride

2.6.1 RNA, DNA and Protein Isolation

2.6.1.1 RNA Isolation

Total RNA was extracted from placental tissue and cultured trophoblast cells using Tri Reagent ™ (Sigma), which is based on the single step method previously reported (Chomczynski and Sacchi, 1987; Chomczynski, 1993). All steps were carried out in certified RNase free micro centrifuge tubes (Anachem Ltd., UK), using RNase free reagents dispensed from RNase free aerosol resistant pipette tips (BDH).

Tissue chunks were ground to a fine powder under liquid nitrogen then added to the Tri Reagent (1ml per 100mg tissue) and further homogenised using a Polytron homogeniser (Kinematica, Switzerland). The resulting solution was spun at 12,000*g* for 10min at 4°C to remove insoluble material, and the supernatant transferred to a fresh 2ml micro centrifuge tube. Cells were lysed directly on the culture surface using 1ml tri reagent per

$8x10^6$ cells, and the solution transferred to a 2ml micro centrifuge tube. The procedure for both tissue chunks and cultured cells was identical from this point. Once the cells had been allowed to stand in 1ml Tri Reagent for 5min at room temperature, 0.2ml chloroform (BDH) was added to the tube, which was shaken vigorously for 15sec and allowed to stand at room temperature for approximately 10min. The resulting mixture was spun at 12,000*g* for 15min at 4°C, which caused the formation of three layers. The upper aqueous phase containing the RNA was transferred to a fresh tube while the lower red coloured organic layer and the white interphase were retained for DNA and protein isolation. Isopropanol (0.5ml) (BDH) was added to the aqueous phase, mixed and incubated at room temperature for 5-10min to allow the RNA to precipitate. This was spun at 12,000*g* for 10min at 4°C to allow the RNA precipitate to form a pellet. The RNA pellet was washed using 1ml 75% ethanol, briefly air dried, and resuspended in diethylpyrocarbonate (DEPC, Sigma) treated water. To confirm the purity and estimate the quantity of RNA isolated, the absorbance of the RNA solution was checked, suspended in TE buffer, at 260nm and 280nm using a Gene Quant II spectrophotometer (Pharmacia Biotech). The 260nm/280nm ratio was generally between 1.8 and 2.2, indicating pure RNA. The quantity of RNA isolated was estimated by multiplying the absorbance reading at 260nm by 40 to give the concentration of RNA in the cuvette in μg/ml. Denaturing gel electrophoresis was used to determine the integrity of isolated RNA. A small sample of RNA mixed 1:2 in RNA loading buffer was heated to 65°C for 10min then loaded onto a 1.2% agarose, denaturing formaldehyde (0.6M) gel in MESA buffer. An RNA marker (0.2-10Kb) was also included on the same gel to determine if the two visible rRNA 28s and 18s bands were of the correct size (5333b and 2366b respectively). A representative gel is illustrated in figure 2.6.1.1. Isolated RNA was stored at –70°C, precipitated in 70% ethanol, 0.083M sodium acetate (pH5.2).

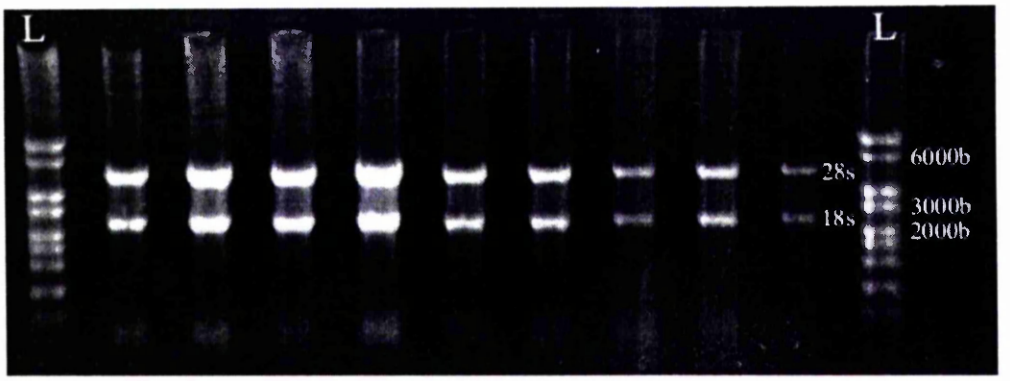


Figure 2.6.1.1 *Representative RNA check gel with 9 different RNA samples extracted from term placental trophoblast cultures. L=RNA ladder. 28s and 18s bands are clearly visible and are of the correct size.*

2.6.1.2 DNA Extraction

DNA was precipitated from the lower organic phase and interphase, retained from the RNA isolation procedure, by addition of 0.3ml absolute ethanol per 1ml tri reagent used. This was mixed and allowed to stand at room temperature for 2-3min before centrifugation at 2,000*g* for 5min at 4°C. The supernatant was removed and retained for subsequent protein isolation. The DNA pellet was washed twice in 0.1M sodium citrate, 10% ethanol solution, allowing the DNA to stand in this solution for 30min for each wash. The pellet was then suspended in 75% ethanol and allowed to stand for 10-20min at room temperature before freeze drying under a vacuum for 10min and dissolving in 8mM sodium hydroxide.

2.6.1.3 Protein Isolation

Protein was precipitated from the phenol-ethanol supernatant retained from the DNA isolation procedure by addition of 1.5ml isopropanol per 1ml tri-reagent used. The tubes were incubated at room temperature for 10min before centrifugation at 12,000*g*. The supernatant was discarded and the protein pellet washed 3 times in 0.3M guanidine hydrochloride/95% ethanol. The pellet was allowed to stand in this solution for at least 20min for each wash step. The protein pellet was suspended in absolute ethanol and allowed to stand at room temperature for 20min before centrifuging at 7,500*g* for 5min at 4°C. The pellet was then freeze dried under vacuum for 10min and dissolved in 1% SDS solution. The pellet obtained from cultured trophoblasts was suspended in 200µl 1% SDS solution and stored at -40°C until being assayed for total protein content (section 2.4.1).

2.6.2 Development of hCG mRNA Assay

In order to quantify the amount of hCG mRNA in either cultured trophoblasts or placental tissue chunks, a method was developed based on the Quantikine® mRNA assay kit (R&D Systems Europe Ltd.). This is a microplate-based assay that quantifies specific mRNA at much lower levels than required for northern blotting and was first described as a method of quantifying specific ribosomal RNA from bacterial cells (Wicks et al. 1998). The outline of the method is illustrated in figure 2.6.2. Specific probes for GAPDH were available from R&D Systems, however specific probes for hCG had to be designed and the assay system optimised for their use.

2.6.2.1 Design of Target Specific Probes

From the Genbank mRNA sequence of the hCG β_5 subunit, two probes were designed to hybridise with either end of the mRNA (fig 2.6.2.1).

The first, directed against the 5' end of the mRNA was 34 bases in length and had the sequence:

5' CAT CTC CAT CCT TGG TGC GTC CCC TGC CTT GTC T 3'.

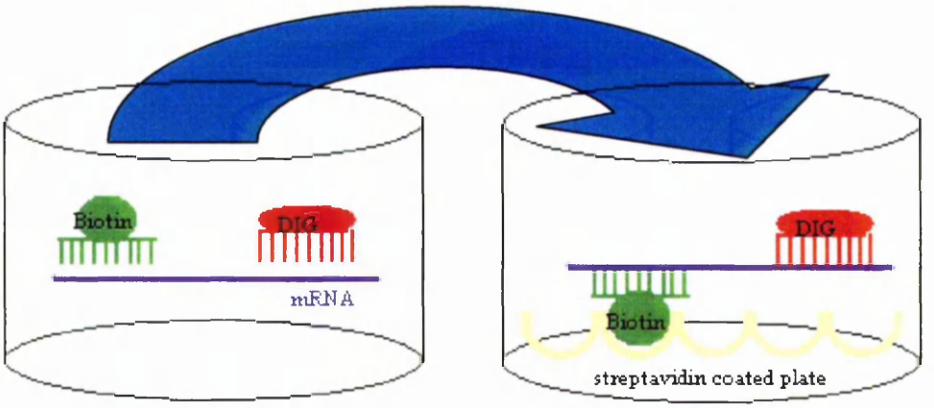
The second, directed against the 3' end of the mRNA was 39 bases long and had the sequence:

5' GCG GAT TGA GAA GCC TTT ATT GTG GGA GGA TCG GGG TGT 3'.

The 34 base probe was 5'biotin labelled, while the other was 5'digoxigenin labelled, and were both obtained from VH Bio Ltd. (UK). PCR was used to determine the specificity of the probes, which meant a complementary probe was required to act as a primer, which had the sequence:

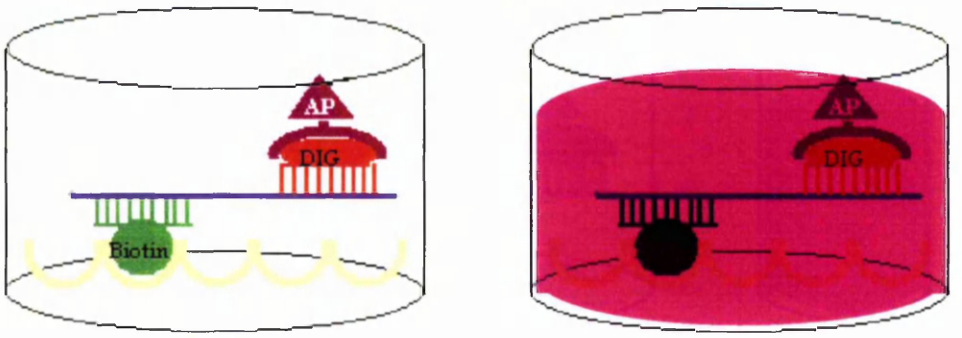
5' AGA CAA GGC AGG GGA CGC ACC AAG GAT GGA GAT G 3'.

This probe was 5'biotin labelled and was also used as a control to exclude the presence of contaminating DNA. To check for the formation of secondary structures within or between probes, the sequences were analysed using Net Primer (www.premierbiosoft.com). Cross reactivity of the probes with other genes was excluded by carrying out a BLAST search (www.ncbi.nlm.nih.gov/blast/) for each probe.



1. Total RNA and target specific labelled probes are hybridised at 65/70°C for 1h.

2. Hybridisation mixture is transferred to a streptavidin coated microtitre plate and incubated at room temperature for 1h.



3. Plate is washed and alkaline phosphatase labelled anti-digoxigenin antibody is added to plate and incubated at room temperature for 1h.

4. Plate is washed and substrate is added to plate and incubated at room temperature for 30min until colour develops.

Figure 2.6.2 *Basis of plate based hCG mRNA quantification method. The first well is that of a standard microtitre plate; while the following three depict one well of a streptavidin coated capture plate that will bind biotin. Digoxigenin (DIG) labelled probe is shown in red and biotin labelled probe is shown in green. Alkaline phosphatase (AP) labelled anti DIG antibody is coloured purple.*

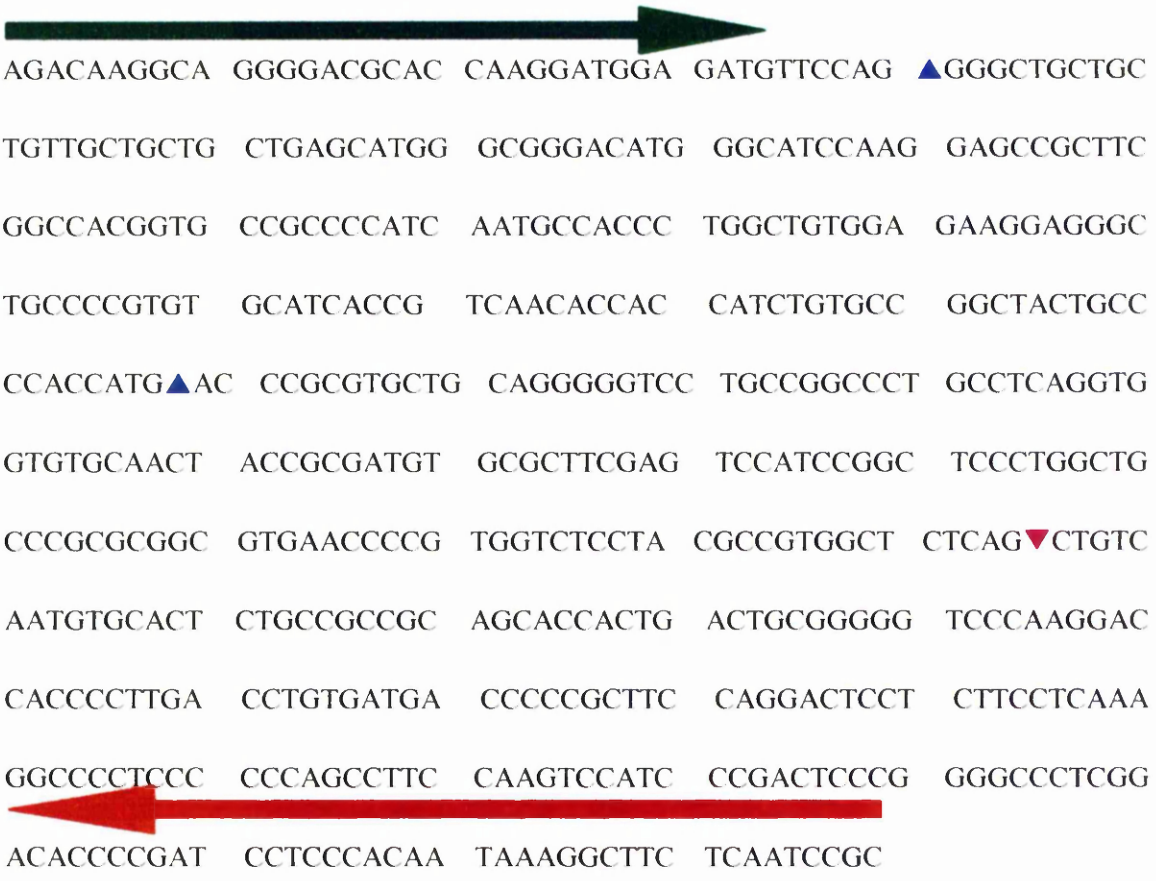


Figure 2.6.2.1 *hCG mRNA sequence with forward and reverse probes/primers indicated by green and red arrows. Intron exon boundaries are indicated by ▲ and AluI restriction enzyme cleavage site by ▼.*

2.6.2.2 Reverse Transcriptase Polymerase Chain Reaction (Oligo DT Method)

Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using the TrueSprinter™ RT-PCR kit (Hybaid UK) on total RNA isolated from three different placentae. This method involves the use of oligo DT primers and was chosen because this is particularly good for amplifying long mRNA transcripts. The buffers supplied with the kit had the following composition:

10x RT-Buffer	**10x PCR-Buffer**
500mM Tris/HCl (pH8.3)	500mM Tris/HCl (pH9.1)
750mM potassium chloride	140mM ammonium sulphate
30mM Magnesium chloride	17.5mM magnesium chloride

For each RT reaction, 1μl RNA (5μg, denatured at 70°C for 5min), 0.5μl RNase inhibitor (40U/μl), 1μl oligo $d(T)_{15}$ primer (20μM), 2μl 10x RT-Buffer, 4μl dithiothreitol (DTT, 50mM), 0.4μl deoxynucleoside triphosphate mix (dNTP-mix, 40mM), 10.1μl DEPC treated water and 1μl reverse transcriptase (50U/μl) were assembled in a microcentrifuge tube, on ice. The tube was then incubated at 42°C for 45min, then 70°C for 10min. The resulting cDNA was used in the PCR reaction using the $hCG\beta_5$ primers. For each PCR reaction, 2μl cDNA, 5μl 10x PCR-buffer, 3μl forward primer (300nM), 3μl reverse primer (300nM), 1μl dNTP-mix, 30.5μl water and 0.5μl Taq/Pwo mix were assembled on ice. A negative control using water instead of cDNA was also included. The PCR reaction was carried out on a Hybaid OmniGene thermal cycler with an initial denaturation step of 94°C for 5min, then 30 cycles of 94°C 1min, 65°C 1min and 72°C 1min with a final 10min 72°C elongation step. To confirm that the PCR reactions amplified the correct region of cDNA, the PCR product was digested using Alu I restriction enzyme (recognition sequence 5' AG▼CT 3', Promega, UK) known to cut the 539bp product into two fragments of 345bp and 194bp. For one digest, 15.45μl water, 3μl 10x buffer (60mM Tris-HCl (pH 7.5), 600mM sodium chloride, 60mM magnesium chloride and 10mM DTT), 0.3μl BSA (1mg/ml), 7.5μl PCR product and 3.75μl Alu I restriction enzyme (10U/μl) were combined and incubated at 37°C for 3h. A negative control digest using no enzyme was included for each sample. The digest products (mixed with DNA loading buffer) were visualised by gel elctrophoresis on 1.5% agarose gel in TAE buffer containing 0.4μg/ml ethidium bromide. A DNA marker (pGEM, Promega, UK) was also included on the gels.

2.6.2.3 hCG mRNA Assay Procedure

A Quantikine® mRNA base kit was purchased from R&D systems, which included the following items:

A hybridisation 96 well microplate
A streptavidin coated 96 well microplate
21ml each of calibrator and sample diluent (buffered protein solutions)
21 ml anti-digoxigenin conjugate (alkaline phosphatase conjugated polyclonal anti-digoxigenin antibody solution)
100ml 10x wash buffer concentrate
Substrate (NADPH) and 7ml buffered diluent
Amplifier (alcohol dehydrogenase and diaphorase) and 7ml amplifier diluent (ethanol and INT-violet)
6ml stop solution (2N sulphuric acid)
Plate sealers
Float collar (allows microplate to float in water bath)

Biotin and digoxigenin labelled probes were suspended in a diluent made up in DEPC treated water, so that once probes and sample were added to the wells of the hybridisation plate, probes were at a final concentration of 0.025μM in 2.5xDenhardt's solution (0.05% solution of BSA, ficoll and polyvinylpyrrolidine, made from 50x Denhardt's, Sigma) with 0.125mg/ml sheared salmon sperm (CP Labs). This solution was derived from the original paper describing this method of RNA quantification (Wicks et al. 1998). An arbitrary standard curve of placental RNA was constructed by diluting total RNA, isolated in bulk from term placental chunks, in calibrator diluent. The eight point standard curve ranged from 0-30μg RNA per well.

The hybridisation plate was washed twice using the supplied wash buffer, before 50μl hCG specific probes were added to the wells of the plate. Next, 150μl standard or appropriately diluted sample RNA was added in duplicate to the wells of the plate, which was then sealed, placed in a float collar and incubated at 70°C in a water bath for 60min. The streptavidin-coated plate was washed twice before 150μl of the RNA probe mixture was transferred from the hybridisation plate to the streptavidin plate. This was incubated at room temperature for 60min on a plate shaker before washing 4 times with

the supplied buffer. Next, 200µl anti-digoxigenin conjugate was added to the wells, which were sealed and incubated at room temperature for 60min on a plate shaker. The plate was washed 6 times as before, then 50µl substrate solution was added to each well, and the plate incubated at room temperature for 60min on a plate shaker. An equal volume (50µl) of amplifier solution was added to each well, and the plate incubated at room temperature for 30min on a plate shaker. Finally, 50µl stop solution was added to each well, and the absorbance determined at 492nm corrected at 690nm. The results were extrapolated by linear regression from the standard curve plotted on a log/log scale.

Probes specific for GAPDH were obtained from R&D Systems and these assay results used as a control against which the hCG results could be standardised. The protocol for this assay was the same as that outlined for the hCG assay except that probes were supplied in a diluent rather than being diluted in the Denhardt's/salmon sperm diluent, and hybridisation was carried out at 65°C.

2.6.3 Northern Blotting

2.6.3.1 Gel Electrophoresis and Transferring RNA

RNA samples (1, 2, 5, 10 or 20µg) were mixed 1:2 with RNA loading buffer and loaded into the wells of a 1% agarose, denaturing formaldehyde (0.6M) gel in MESA buffer. An RNA marker (0.2-10Kb) was also included on the same gel to provide an indication of the size of the final bands. The gel was run at 80 volts for approximately 1h until good separation of the RNA marker could be seen. After running, the gel was washed in DEPC treated water to remove the formaldehyde and then soaked in 0.05M sodium hydroxide to partially hydrolyse the RNA. It was washed again in water before soaking for 45min in 20xSSC. The RNA was transferred to a nylon membrane (Hybond-N, Amersham Life Science, UK) by capillary blotting overnight using 20xSSC buffer. RNA transfer was confirmed by viewing the gel on a UV transluminator to ensure no RNA remained. The membrane was briefly washed, then baked at 80°C for 3½h to fix the RNA to the membrane.

2.6.3.2 Probe Preparation

The following buffers were used during probe preparation:

STET Buffer (pH 8)	**10x Restriction Digest Buffer**
8% sucrose	100mM Tris-HCL (pH7.5),
5% triton-X-100	500mM potassium chloride
50mM EDTA	70mM magnesium chloride
50mM Tris	10mM DTT

E.Coli containing a plasmid with beta-hCG5 DNA sequence (Talmadge et al. 1984) were obtained from the American Type Culture Collection and cultured for 24h in LB-broth (Gibco BRL) containing 50μg/ml ampicillin. The plasmid (pBR322) was extracted using the triton-lysis mini-prep method. The cells were suspended in 350μl ice cold STET buffer to which 25μl freshly prepared lysozyme (10mg/ml) was added. This mixture was boiled for 90sec then spun at 2,500*g* for 15min. The supernatant was transferred to a fresh tube and the centrifugation/removal of supernatant procedure was repeated 8-10 times until as much supernatant as possible was collected. The DNA was extracted from the supernatant by addition of ½ volume phenol:chloroform mixture (1:1) then centrifuging at 1,200*g* and collecting the supernatant. The DNA was precipitated from this solution by addition of isopropanol and 3M sodium acetate than incubating in dry ice for 30min. The DNA pellet was washed with 95% ethanol and re-suspended in water.

The resulting DNA was digested using Kpn I (recognition sequence 5' G GTA$^{\blacktriangledown}$CC 3', Promega, UK) and Sma I (recognition sequence 5' CCC$^{\blacktriangledown}$GGG 3', Promega, UK) restriction enzymes to elucidate the structure of the construct, and determine which section should be used as a probe. The digests comprised 15.75μl water, 2.5μl 10xbuffer, 0.25μl BSA (1mg/ml), 5μl DNA, 3μl enzyme. These were incubated for 1h at 37°C in the case of Kpn I and 1h at 25°C in the case of Sma I. The digest products were mixed with 5μl DNA loading buffer and visualised by gel electrophoresis (fig 2.6.3.2a) on a 1% low melting temperature agarose gel (NuSieve GTG agarose, Flowgen) in TAE buffer. From these results the structure of the construct could be established (fig 2.6.3.2b) A large scale Sma I digest was carried out to produce enough of the probe to be excised from the gel and used in northern blotting. The excised piece of gel was weighed and 3μl water added to it for each μg of gel and boiled for 2min.

The probe was ^{32}P labelled using the RmT random primer labelling kit (Stratagene). Probe solution (42µl) was boiled for 5min before 5µl deoxycytidine-5'-triphosphate [α-^{32}P] (dCTP) and 3µl magenta DNA polymerase were added and incubated at 37°C for 20min. Labelled probe was separated from unincorporated nucleotides using the MicroSpin G-50 Column (Amersham Parmacia Biotech). The column was pre-spun for 1min at 735g and transferred into a fresh supporting 1.5ml tube. The probe mixture was carefully layered on to the sephadex in the column before centrifugation at 735g for 2min. The purified sample was collected in the supporting tube and used to probe the fixed membrane

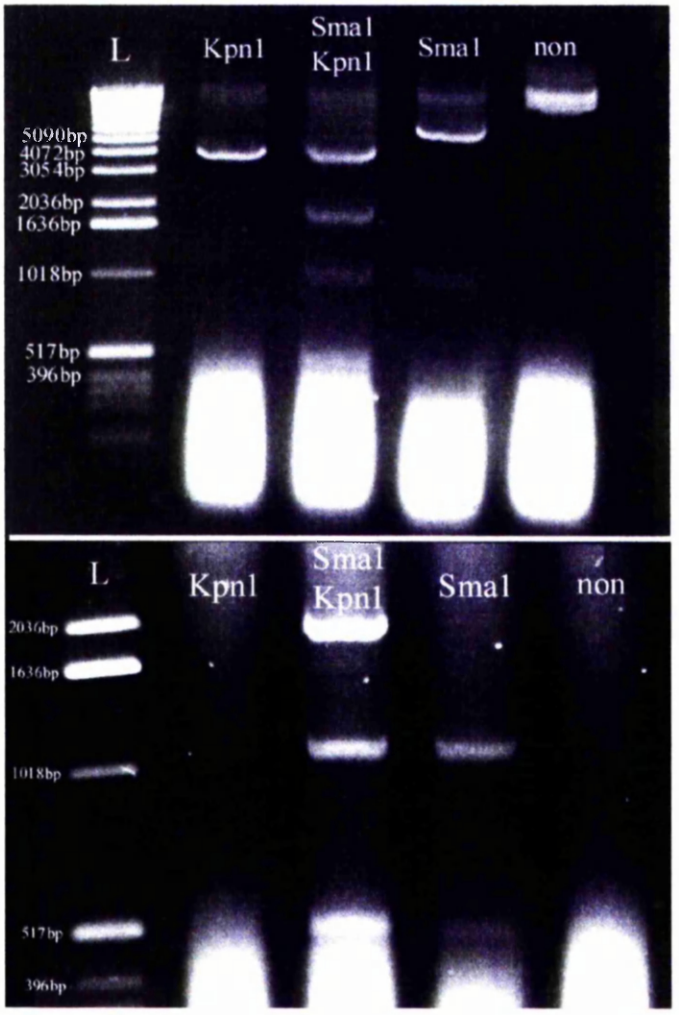


Figure 2.6.3.2a *Results of digesting hCG plasmid with Kpn1, Sma1&Kpn1, Sma1 and undigested plasmid (non). A DNA marker was also run (L). Both photographs are of the same gel, with the lower showing a higher magnification to allow visualisation of the 500bp fragment. Approximate fragment sizes generated by each combination were:*

Kpn1 - 2 at 4.2Kb;

Kpn1&Sma1 – 4.2Kb, 2Kb, 1Kb, 2 at 500bp;

Sma1 – 7Kb, 1Kb, 500bp.

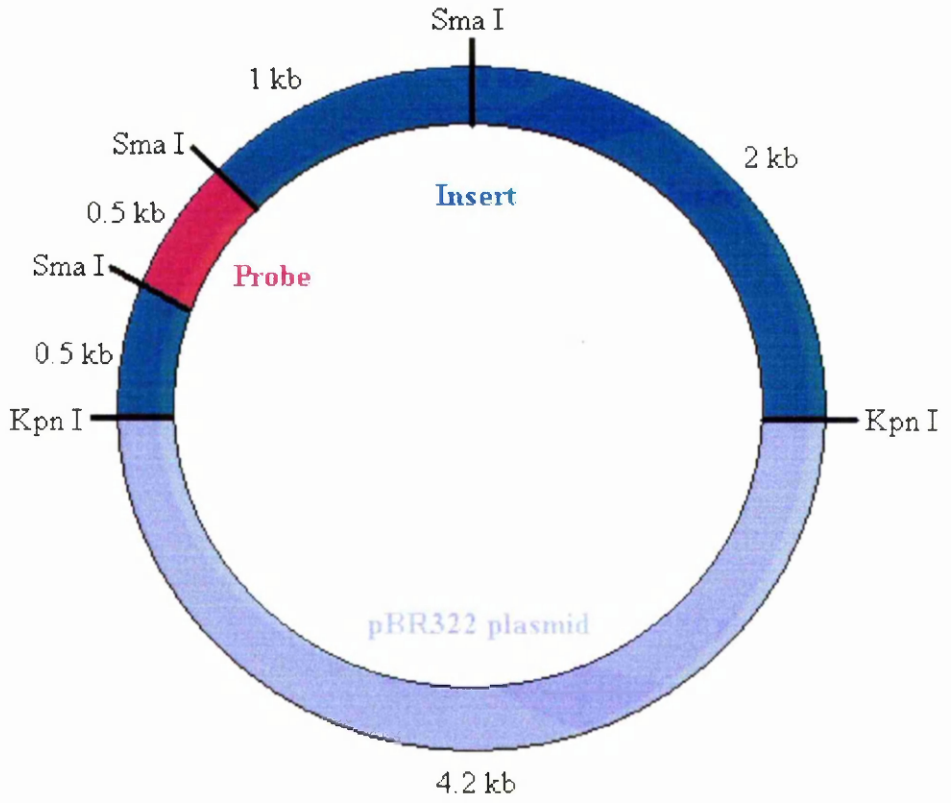


Figure 2.6.3.2b *Graphical representation of pBR322 plasmid and insert, highlighting the area that was subsequently used as a northern blotting probe. This diagram was constructed using the results illustrated in fig 2.6.3.2a.*

2.6.3.3 Hybridisation

Following 3½h of baking, the membrane was soaked in DEPC treated water for 2min then placed in a sealing glass tube to which 5ml preheated QuikHyb hybridisation solution (Stratagene) was added. The membrane was pre-hybridised for 20min at 68°C in a rotating oven. Meanwhile, 150µl sheared salmon sperm DNA was added to the probe and boiled for 5min. This was added to 1ml QuikHyb, which was then added to the hybridisation tube. The membrane was hybridised for 2h at 68°C. Following hybridisation, the membrane was washed at room temperature in 2xSSC, 1%SDS then 1xSSC, 1%SDS and 0.1xSSC, 0.1% SDS. The membrane was then wrapped in plastic and placed on Kodak autoradiography film in a light proof cassette containing intensifying screens and exposed at -40°C for up to 96h. Film was developed in a Fuji RGII x-ray film processor.

2.6.4 DNA Assay

To standardise RNA measurements in each sample, the total DNA content was measured to give an indication of cell number. This was carried out by a method modified from that of Labarca and Paigen (1980). The fluorochrome Hoechst 33258 (bisbenzimide) preferentially binds the minor groove of DNA (preferring A-T rich regions) under high salt conditions, allowing the concentration of DNA to be determined in the presence of proteins, RNA, nucleotides and buffer reagents. When bound to DNA, Hoechst 33258 dye has an excitation peak at 360nm and an emission peak at 460nm. Because Hoechst 33258 preferentially binds to A-T sequences, a standard curve should be constructed from DNA with a similar A-T content to the samples.

Before commencing the assay, DNA standards (1mg/ml, 100μg/ml and 10μg/ml) were prepared using DNA type XIII from human placenta (Sigma) in distilled deionised water. A 2μg/ml solution of Hoechst 33258 was prepared containing 10mM Tris, 1mM EDTA and 2M sodium chloride in distilled deionised water. The DNA assay was carried out in black wall microtitre plates (Jencons, UK) suitable for fluorescence-based assays. Each well received 5μl sample or the appropriate quantity of DNA standard (see table 2.6.4) in duplicate. Next, 200μl Hoechst solution was added to each well, which was mixed thoroughly before the plate was transferred to a Wallac Victor^2 1420 fluorimeter (Perkin Elmer, Finland). The excitation filter was set to 355nm, and the emission filter was set to 460nm. The fluorescence emitted from each well was determined and the concentration of DNA in the samples extrapolated from the standard curve using MultiCalc software.

Total DNA per well	DNA stock solution used	Volume of DNA standard
2μg	1mg/ml	2μl
1μg	100μg/ml	10μl
0.5μg	100μg/ml	5μl
0.1μg	10μg/ml	10μl
0.05μg	10μg/ml	5μl
0.02μg	10μg/ml	2μl

Table 2.6.4 *Construction of DNA standard curve using previously prepared stock solutions.*

2.6.5 Taq Man® Real Time PCR

Real time PCR allows the quantitative analysis of mRNA for specific genes using a small sample size. The RNA sample was firstly reverse transcribed using random primers. Real time PCR analysis was then carried out using the ABI Prism® 7700 sequence detection system. Standard PCR primers are used in the reaction, along with target specific probes that contain a fluorophore (5' TET or 5' FAM) and a fluorogenic quencher (3' TAMRA). As long as the fluorophore and quencher are both linked to the probe, fluorescence is quenched. During the PCR amplification, the probe binds to the complementary cDNA strands and the exonuclease activity of the Taq polymerase cleaves the fluorophore from the probe. This means that the fluorophore is no longer quenched by TAMRA so the fluorescence can now be measured. The amount of fluorescence is directly proportional to the amount of target DNA accumulation during the PCR cycles and is recorded by the TaqMan® machine. After the PCR reactions have been completed amplification curves are created for each sample where the number of PCR cycles is plotted against the apparent amount of PCR product at that cycle (ΔRn value). These amplification plots can be compared for each sample and the gene of interest can be quantified relative to a housekeeping gene by comparing the number of cycles each sample requires to reach the threshold cycle (Ct) that is the point at which the amplification curve reaches a plateau.

2.6.5.1 *Design of Target Specific Primers and Probes*

Primers and probes specific to hCGβ mRNA and the mRNA for the housekeeping gene, cyclophilin A (PPIA) previously described by Frendo *et al* (2000) were obtained from VH Bio Ltd. (UK). The hCG probe was labelled with a 5'-FAM fluorescent label and the PPIA probe was labelled with 5'-TET. Both probes had a 3' TAMRA fluorescence quencher label. The specificity of the probes was confirmed by carrying out a BLAST search (www.ncbi.nlm.nih.gov/blast/). To further confirm the specificity of the probes a negative control cDNA was used which was known not to express hCG mRNA, and a "no cDNA" control (water) was also included in each run. The sequences of probes and primers are detailed in table 2.6.5.1.

Gene	Sequence
hCG β fwd primer	5'-GCTACTGCCCCACCATGACC-3'
hCG β rev primer	5'-ATGGACTCGAAGCGCACATC-3'
hCG β probe	5'-FAM-CCTGCCTCAGGTGGTGTGCAACTACC-TAMRA-3'
PPIA fwd primer	5'-GTCAACCCCACCGTGTTCTT-3'
PPIA rev primer	5'-CTGCTGTCTTTGGGACCTTGT-3'
PPIA probe	5'-TET-AGCTCAAAGGAGACGGCCCA-TAMRA-3'

Table 2.6.5.1 *Sequences of probes and primers used in TaqMan® real time PCR analysis.*

2.6.5.2 *Reverse Transcriptase Polymerase Chain Reaction (Random Primer Method)*

RT-PCR was carried out using the Reverse Transcription System (Promega) on total RNA isolated from placental trophoblast cultures. This method involves the use of random primers and was chosen because neither end of the mRNA transcripts is preferentially transcribed. The supplied RT buffer had the following composition:

10x RT-Buffer (pH9)

100mM Tris-HCl

500mM Potassium chloride

1% Triton-X-100

For each RT-PCR reaction, 2µl RNA (2µg, denatured at 70°C for 5min), 8µl magnesium chloride (25mM), 1µl RNasin RNase inhibitor (40U/µl), 2µl random primers (0.5µg/µl), 4µl 10x RT-Buffer, 4µl dNTP-mix (10mM), 17.5µl nuclease free water and 1.5µl reverse transcriptase (20U/µl) were assembled in a microcentrifuge tube, on ice. The tube was then incubated at room temperature for 10min, 42°C for 30min, and then 95°C for 5min. The resulting cDNA was stored at -40°C until used in TaqMan® analysis.

2.6.5.3 Real Time PCR Procedure and Optimisation

The real time PCR reaction was assembled in a 96 well PCR reaction plate with optically clear sealing strips. TaqMan® universal PCR master mix was obtained from Applied Biosystems (UK). This is a 2x reaction mix containing all of the components required for the PCR reaction, excluding primers and probes. Both primers and probes were diluted to a concentration of 5pmol/µg. Primer concentrations of 50, 300 and 900nm were tested to find the optimum working concentration. Using this primer concentration, probe concentrations of 25, 50, 75, 100, 125, 150, 175 and 200nm were tested. The optimum primer or probe concentration is determined by finding which gives the lowest Ct value i.e. reaches the threshold cycle fastest, and the highest product yield as determined by the highest ΔRn value. This optimisation procedure was carried out for both the hCG and PPIA primers and probes. Tables 2.6.5.3a and 2.6.5.3b give the Ct and ΔRn values obtained for each primer and probe concentration that was studied. A compromise between the best ΔRn and Ct values usually has to be chosen, and also the practicality of which primer and probe concentrations are easiest to work with if two concentrations give similar results. Optimum primer concentrations for both hCG and PPIA was 300nM and optimum probe concentrations were both 225nM.

For each multiplex PCR reaction, 12.5µl 2x universal PCR master mix, 1.74µl nuclease free water, 1.5µl each fwd and rev primer (final concentration 300nM), 1.13µl each probe (final concentration 225nM) and 2.5µl cDNA was combined in the reaction tube to give a total volume of 25µl. All samples were studied in duplicate. This was transferred to the pre-heated TaqMan® machine and a standard real time PCR reaction was carried out with the following steps. The first step was 50°C for 2min then a

denaturation step of 95°C for 10min, which also triggers the hot start Taq polymerase in the reaction mix. Then 40 cycles of 95°C for 15sec and 60°C for 60sec were carried out. This is a two-step PCR reaction that allows elongation to occur as the temperature in the tube is returning to the annealing temperature.

Fwd primer (nM)	Rev primer (nM)	HCG		PPIA	
		ΔRn	Ct	ΔRn	Ct
50	50	2.0015	18.29	0.256825	22.05
50	300	2.5483	17.98	-0.014061	40
50	900	2.46465	17.945	0.192485	23.24
300	50	1.36035	17.13	0.32085	21.475
300	**300**	**2.26395**	**16.585**	**0.40897**	**21.89**
300	900	2.49335	16.625	0.41286	22.28
900	50	0.009726	40	0.191795	21.855
900	300	2.1004	16.45	0.465275	21.74
900	900	2.7973	16.345	0.461495	21.92

Table 2.6.5.3a *Primer concentrations tested for both hCG and PPIA primers and the resulting ΔRn and Ct values obtained. The primer concentrations that were chosen for use are highlighted in bold text.*

Probe (nM)	hCG		PPIA	
	ΔRn	Ct	ΔRn	Ct
25	1.2283	17.36	0.19911	23.38
50	1.42985	17.335	0.25467	22.99
75	1.97975	17.12	0.289475	22.665
100	2.01445	17.145	0.33488	22.6
125	2.5742	16.935	0.43974	22.205
150	2.6911	16.825	0.49993	21.655
175	2.44585	17.069	0.486405	21.92
200	2.7787	16.9	0.579745	21.985
225	**2.94035**	**16.79**	**0.57775**	**21.54**

Table 2.6.5.3b *Probe concentrations tested for both hCG and PPIA primers and the resulting ΔRn and Ct values obtained. The probe concentrations that were chosen for use are highlighted in bold text.*

2.6.5.4 *TaqMan® Data Analysis*

In order to express the hCG mRNA results relative to both the standard calibrator sample and the quantity of housekeeping gene PPIA mRNA, results were expressed as $2^{-\Delta\Delta Ct}$. This is calculated for each sample using the average Ct value from the duplicate reactions for both hCG and PPIA. Firstly the ΔCt value for each sample was calculated by taking the reference (PPIA) Ct away from the target (hCG) Ct ($\Delta Ct=Ct_{target}-Ct_{ref}$). Next the ΔΔCt for each sample was calculated by taking the ΔCt value for the calibrator sample away from the ΔCt for each of the individual samples ($\Delta\Delta Ct=\Delta Ct_{sample}-\Delta Ct_{calibrator}$). Finally the ΔΔCt value of each sample was used to calculate $2^{-\Delta\Delta Ct}$. This is the value that was used to represent the quantity of hCG mRNA present in the samples corrected against the housekeeping gene, and relative to the calibrator sample. For the calibrator sample, this value should be approximately 1.0.

2.7 Statistical Methods

2.7.1 Coefficient of Variation (cv)

The coefficient of variation (CV) gives a measure of assay reproducibility and is the standard deviation (SD) expressed as a percentage of the mean (X). The inter and intra assay CV was calculated using the equation:

$$CV=100x\left(\frac{SD}{X}\right)$$

2.7.2 Regression Analysis

This analysis gives an estimation of the relationship between a dependent variable (gestation) and an independent variable (marker level). Various models were used, the equations for which are outlined below. From the regression equation, median marker levels were determined at each week of gestation for use in calculation of MoM values.

Linear:	$y= a +bx$
Quadratic:	$y=a+bx+cx^2$
Growth:	$y=e^{(a+bx)}$
Inverse:	$y=a+(b\div x)$

2.7.3 Medians, Means and Percentiles

2.7.3.1 Medians

The median is the middle value when cases are sorted in ascending order, also called the 50th percentile. If there is an even number of cases, the median is the average of the two middle cases when they are sorted in ascending or descending order. The median is a measure of central tendency and is not sensitive to outlying values and is therefore used when the data set is skewed.

2.7.3.2 Means

The mean gives the central value of a data set. It is calculated by dividing the sum of all cases by the number of cases. This measure is only used in sample groups with an even distribution.

2.7.3.3 10^{th} and 90^{th} Percentiles

The 10^{th} and 90^{th} percentiles are the values below which 10 and 90 percent of cases fall.

2.7.4 Multiples of the Median (MoM)

Control and DS marker levels were converted to multiples of the control median (MoM) at the appropriate week of gestation. This allows for gestational variation in marker levels, and is calculated using the equation:

$$\text{MoM} = \frac{\text{Marker Level}}{\text{Regresses Median Level at Appropriate Gestation}}$$

2.7.5 Measures of Variable Dispersion

2.7.5.1 Standard Deviation

The standard deviation (SD) is a measure of dispersion around the mean, and is described as the square root of the variance. Standard deviation is calculated using the following equation where y=individual case values and N=number of cases.

$$SD = \sqrt{\frac{\Sigma y^2 - (\Sigma y)^2 \div N}{(N-1)}}$$

2.7.5.2 *Standard Error of Mean (sem)*

The standard error of the mean (sem) gives a measure of how much the value of the mean may vary from sample to sample taken from the same distribution. It is calculated by dividing the standard deviation by the square root of the number of cases.

2.7.6 Analysis of Variation Between Sample Groups

2.7.6.1 *Mann-Whitney U Test*

This is a nonparametric test, which is equivalent to the parametric t-test. It tests whether two independent samples are from the same population. The distributions of both groups are analysed by ranking values in ascending order, and the number of times a value in the first group precedes a value in the second group is calculated (U). If one group has a higher proportion of high ranks, then both groups are likely to have different distributions. This is deemed statistically significant if $p<0.05$.

2.7.6.2 *Sign Test*

This is a nonparametric test used with two related samples to test the hypothesis that two variables have the same distribution. The differences between the two variables for all cases are computed and classified as either positive, negative, or tied. If the two variables are similarly distributed, the numbers of positive and negative differences will not be significantly different. If one group has more positive differences, then the variables are likely to have different distributions. This is deemed statistically significant if $p<0.05$.

2.7.6.3 *General Linear Model Univariate (GLM Univariate)*

This test uses regression analysis and analysis of variance (ANOVA) to determine the effect of various factors or variables on one dependant variable. The GLM Univariate tests null hypotheses about the effects of other variables on the means of various groupings of a single dependant variable. Interactions between factors as well as the effects of individual factors can also be studied. This test can be used on balanced (each cell has the same number of cases) or unbalanced models. If the overall F test has shown a significance ($p<0.05$), then differences between the means of individual groupings can be further investigated using a post hoc test.

2.7.6.4 *One-Way ANOVA*

The One-Way ANOVA procedure produces a one-way analysis of variance of the effect of a dependent variable on a single factor (independent) variable. Analysis of variance is used to test the hypothesis that means are equal. This technique is an extension of the two-sample t-test.

2.7.6.5 *Paired Samples T-Test*

The Paired-Samples t-test compares the means of two variables for a single group. Firstly it calculates the differences between values of the two variables for each case and then tests whether the average differs from 0. A difference is deemed significantly different if $p<0.05$.

2.7.6.6 *Wilcoxon Matched Pairs Signed Ranks Test*

This is a nonparametric test, which is equivalent to the parametric paired t-test. This test is used to determine if two related variables have the same distribution. Unlike the Sign Test, information on the magnitude of difference between the paired samples is taken into account and more weight is given to pairs that show large differences than to pairs that show small differences. The absolute differences between pairs are ranked and information on the number of positive differences, negative difference and ties is reported. If there is a significant difference in the distribution of the pairs of variables, $p<0.05$.

2.7.7 Pearson Correlation Coefficient (r)

The degree of linear association between two variables was quantified using this test. Values of the correlation coefficient (r) range from -1 to 1, with a minus answer indicating a negative correlation and a positive answer indicating a positive correlation. The absolute value indicates the strength of correlation, with larger values indicating stronger relationships. If the significance level of the correlation is less than 0.05, then there is a significant association between these variables.

Section 3

Results

3.1 Levels of Inhibins and Growth Factors in Tissues and Fluids

3.1.1 Inhibin-A Levels

3.1.1.1 Placenta

Regressed median control levels of inhibin-A in placental tissue, expressed as pg/μg protein, were calculated using linear regression on $\log_{10}$ transformed data, giving the equation y=0.001758-0.024194x (figure 3.1.1.1a). Actual and regressed control median values are outlined in table 3.1.1.1. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 1.04, while the DS median MoM was significantly elevated to 1.46 (p=0.041). Individual cases are illustrated in figure 3.1.1.1b.

Gestation	Inhibin-A Medians (pg/μg protein)		Activin-A Medians (pg/μg protein)	
(weeks)	Regressed	Observed	Regressed	Observed
14	4.605	3.920	58.126	67.188
15	4.355	8.020	56.655	45.799
16	4.118	4.270	55.221	73.134
17	3.895	4.090	53.824	36.903
18	3.684	3.090	52.461	88.372
19	3.484	3.69	51.134	64.194
20	3.295	5.050	49.840	22.750
21	3.116	/	48.578	/
22	2.947	/	47.349	/
23	2.787	/	46.150	/

Table 3.1.1.1 *Observed and regressed control placental median inhibin-A and activin-A levels at each week of gestation. Results are expressed as a ratio of protein concentration.*

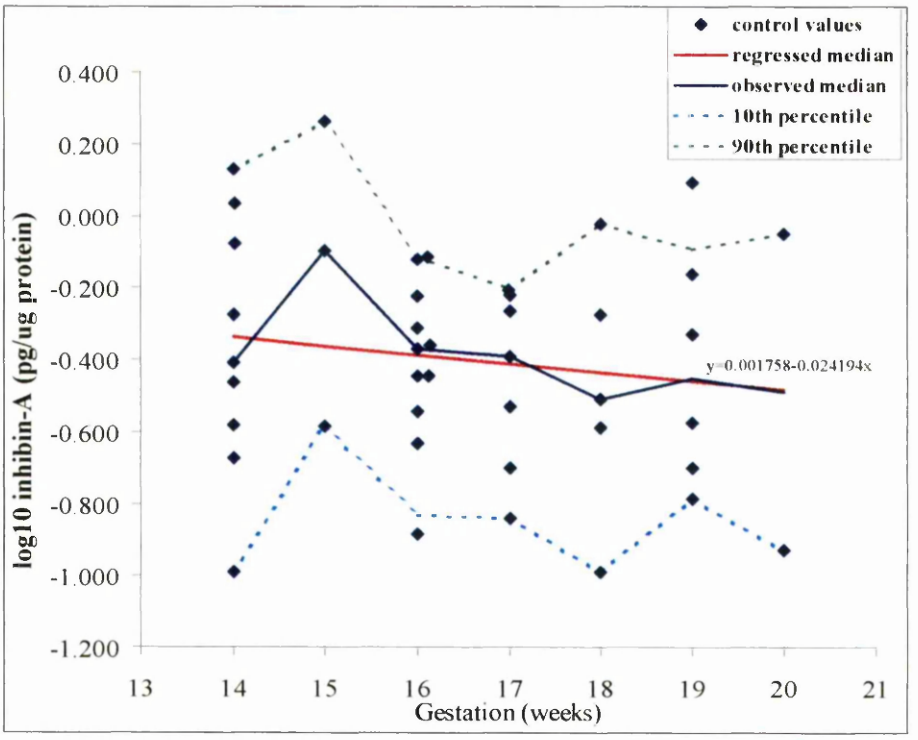


Figure 3.1.1.1a *Individual placental inhibin-A control data points (log_{10} pg/μg protein), with actual and regressed median and 10^{th} and 90^{th} percentile lines plotted.*

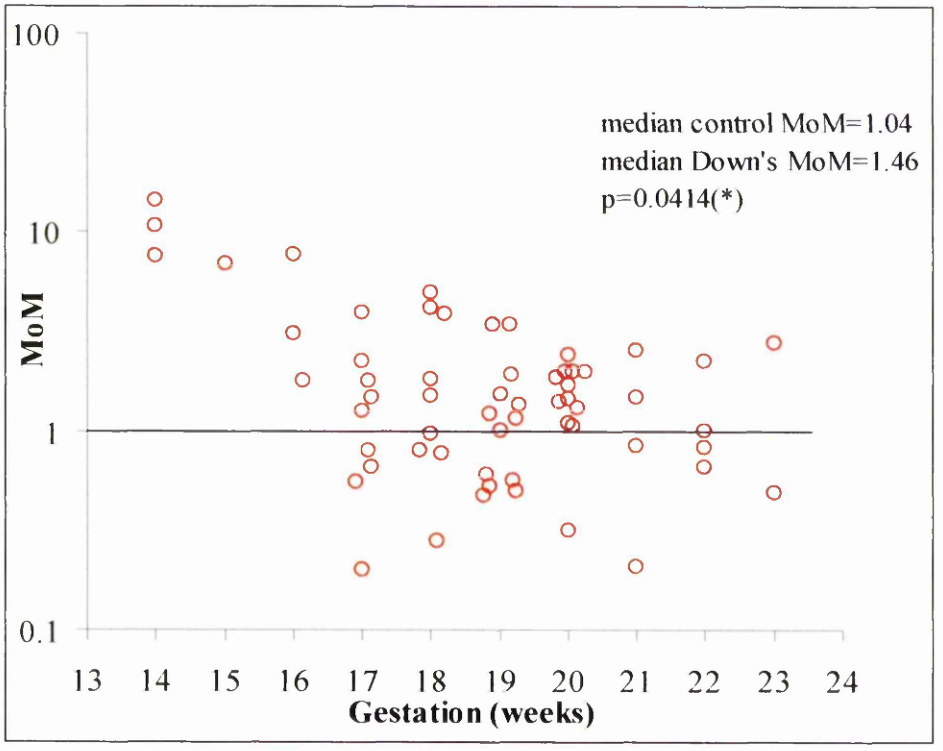


Figure 3.1.1.1b *DS placental inhibin-A MoMs.*

3.1.1.2 *Maternal Serum*

Regressed median control levels of inhibin-A in maternal serum, expressed as pg/ml, were calculated using a quadratic curve fit on $\log_{10}$ transformed data resulting in the equation $y=5.693234-0.38282x+0.010447x^2$ (figure 3.1.1.2a). Actual and regressed control median values are outlined in table 3.1.1.2.

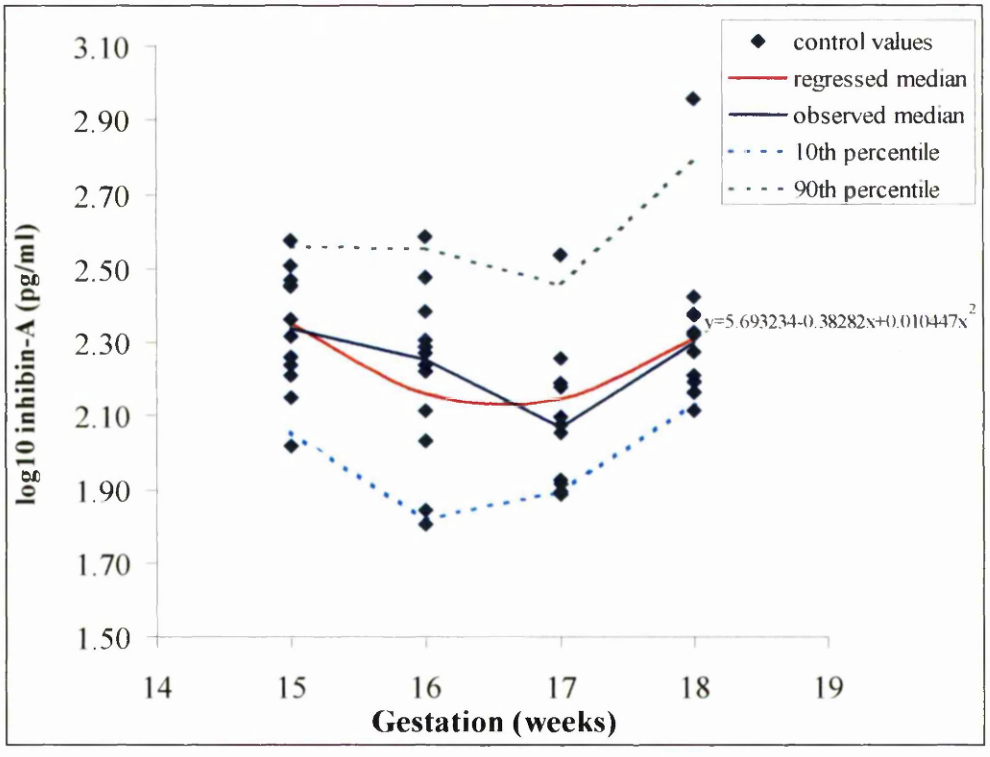


Figure 3.1.1.2a *Individual maternal serum inhibin-A control data points ($\log_{10}$ pg/ml), with actual and regressed median and 10^{th} and 90^{th} percentile lines plotted.*

Gestation	Inhibin-A Medians (pg/ml)		Activin-A Medians (pg/ml)	
(weeks)	Regressed	Observed	Regressed	Observed
15	225.88	219.12	3593.5	3575
16	144.84	180.14	3140.7	3027.5
17	140.16	116.57	3740.7	3907.5
18	204.70	198.34	5393.7	5010

Table 3.1.1.2 *Observed and regressed control serum inhibin-A and activin-A levels at each week of gestation. Results are expressed as pg/ml.*

All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 1.03, while the DS median MoM was significantly elevated to 2.06 ($p<0.0001$). Individual cases are illustrated in figure 3.1.1.2b.

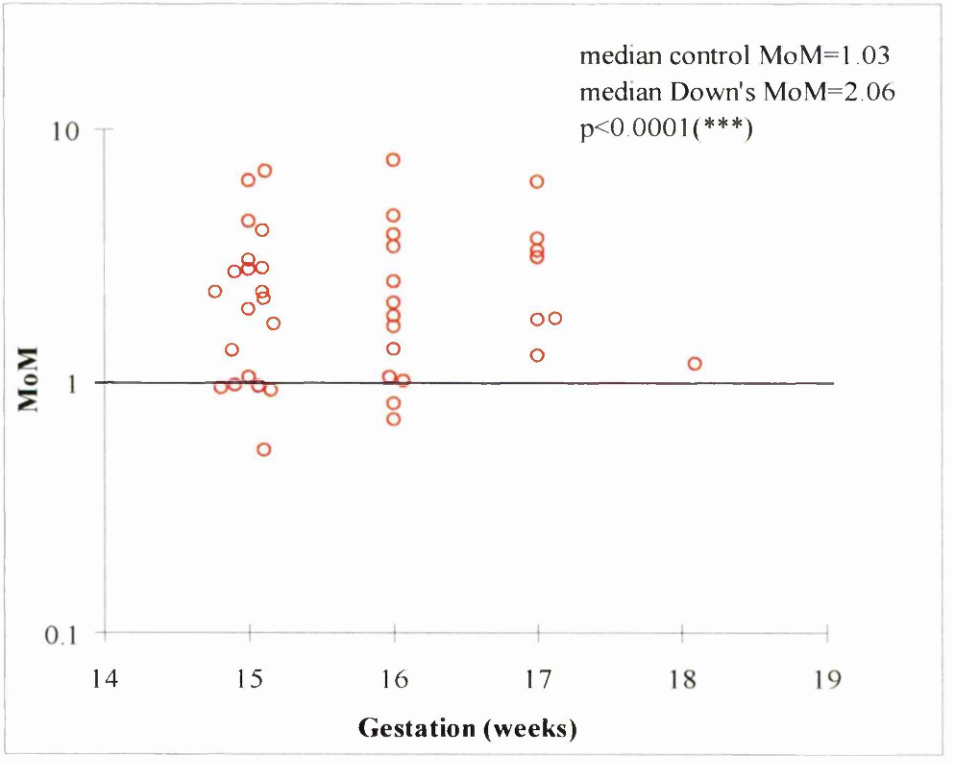


Figure 3.1.1.2b *DS maternal serum inhibin-A MoMs*

3.1.2 Activin-A Levels

3.1.2.1 Placenta

Regressed median control levels of activin-A in placental tissue, expressed as pg/µg protein were calculated using a growth curve fit on non-transformed data, resulting in the equation $y=e^{(2.119098-0.025645x)}$ (figure 3.1.2.1a). Actual and regressed control median values are outlined in table 3.1.1.1. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 1.05, while the DS median MoM was significantly elevated to 1.62 ($p=0.0098$). Individual cases are illustrated in figure 3.1.2.1b.

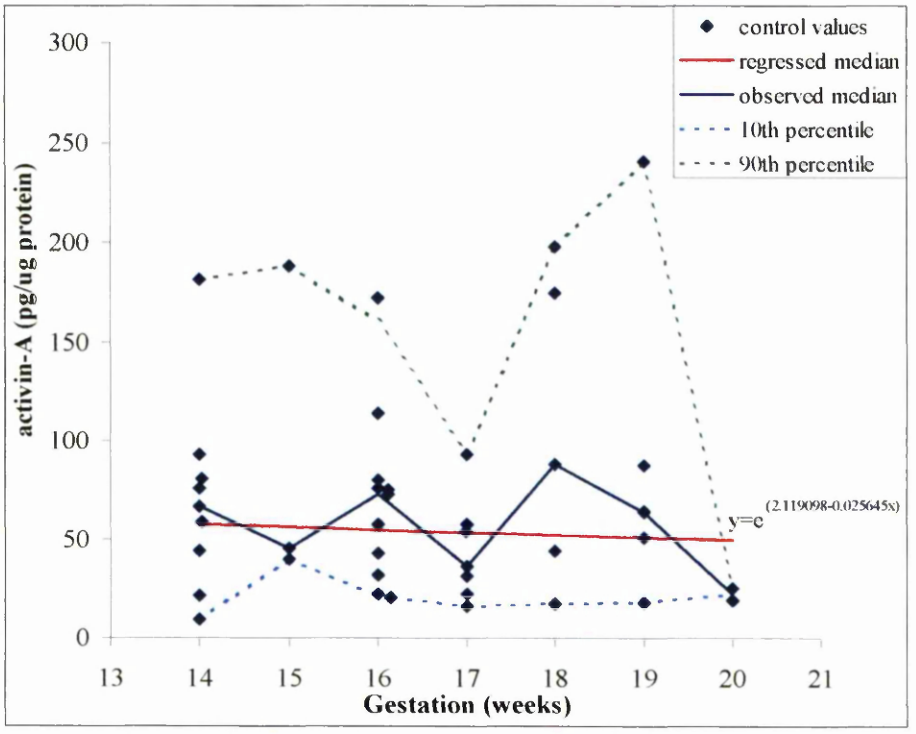


Figure 3.1.2.1a *Individual placental activin-A control data points (pg/μg protein), with actual and regressed median and 10th and 90th percentile lines plotted.*

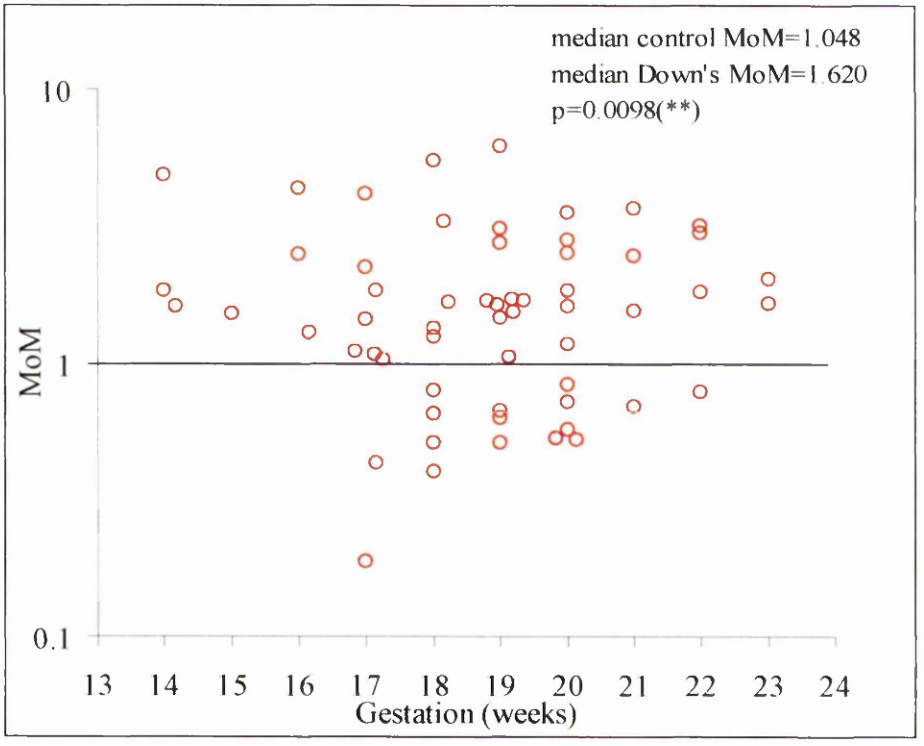


Figure 3.1.2.1b *DS placental activin-A MoMs.*

3.1.2.2 Maternal Serum

Regressed median control levels of activin-A in maternal serum, expressed as pg/ml, were calculated using a quadratic curve fit on log_{10} transformed data resulting in the equation $y=2078.172036x-55.587912x^2-15343.373248$ (figure 3.1.2.2a). Actual and regressed control median values are outlined in table 3.1.1.2. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 1.00, while the DS median MoM was significantly elevated to 1.26 ($p=0.01$). Individual cases are illustrated in figure 3.1.2.2b.

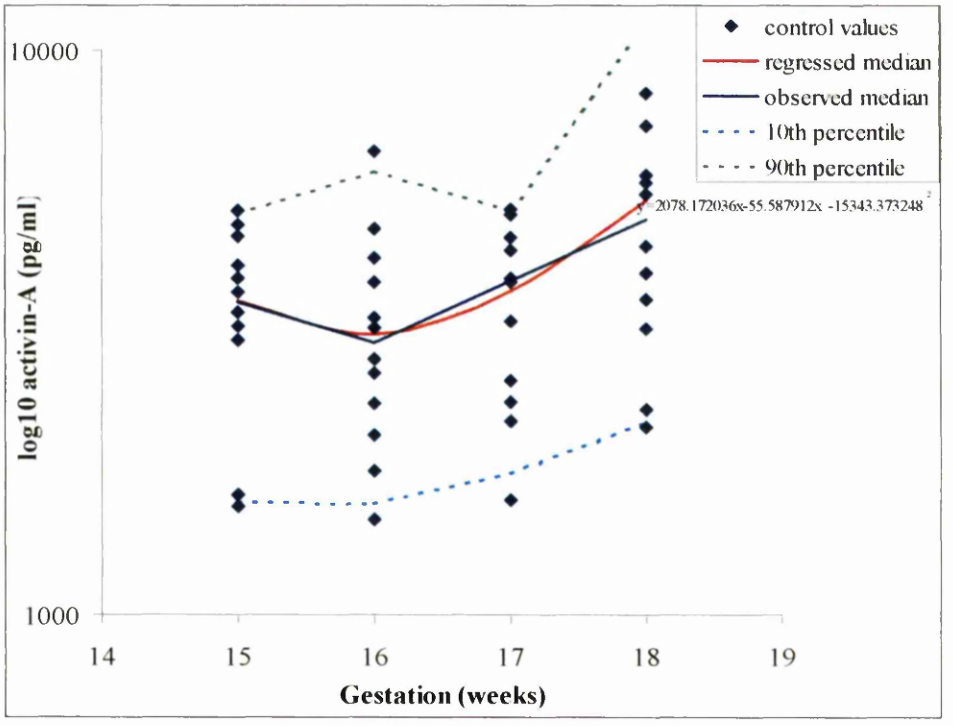


Figure 3.1.2.2a *Individual maternal serum activin-A control data points (log_{10} pg/ml), with actual and regressed median and 10^{th} and 90^{th} percentile lines plotted.*

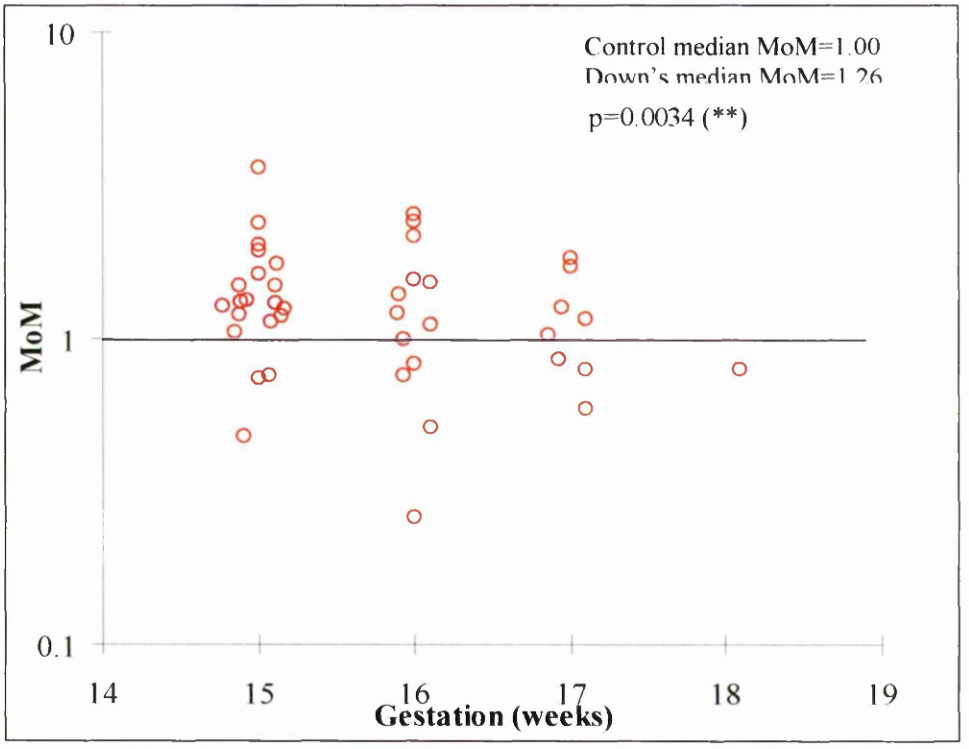


Figure 3.1.2.2b *DS maternal serum activin-A MoMs.*

3.1.3 Placental Expression of Inhibin/Activin Subunits

3.1.3.1 Inhibin/Activin α and β_A Subunit Localisation

Immunohistochemistry shows that the inhibin-α and the inhibin/activin β_A subunits were mainly localised to the trophoblast layer of placental tissues, with both syncytium and cytotrophoblast showing positive reactivity (fig 3.1.3.1a & 3.1.3.1b). Syncytial knots showed positive staining for both subunits. Lighter staining was also noted on the endothelium of blood vessels, particularly in the case of β_A subunit staining. Stromal cells, some of which were characteristic of Hofbauer cells, also showed positive staining for both subunits. No staining was evident in negative control tissue sections (fig 3.1.3.1c).

3.1.3.2 Comparison of Staining Intensity

Two different scorers assessed the staining intensity for both antibodies in the trophoblast layer and stroma of placental villi from 20 matched pairs of DS and control sections. The staining for both antibodies tended to be stronger in the DS sections when compared with controls (fig 3.1.3.1a & 3.1.3.1b). The sign test showed this to be

significantly different only in the case of β_A subunit staining. Table 3.1.3.2 shows scoring intensity for each pair of sections and summary is illustarated in fig 3.1.3.2. Analysis of the effect of scorer on scoring level by one-way ANOVA revealed no significant difference between or within scorer (p=0.7).

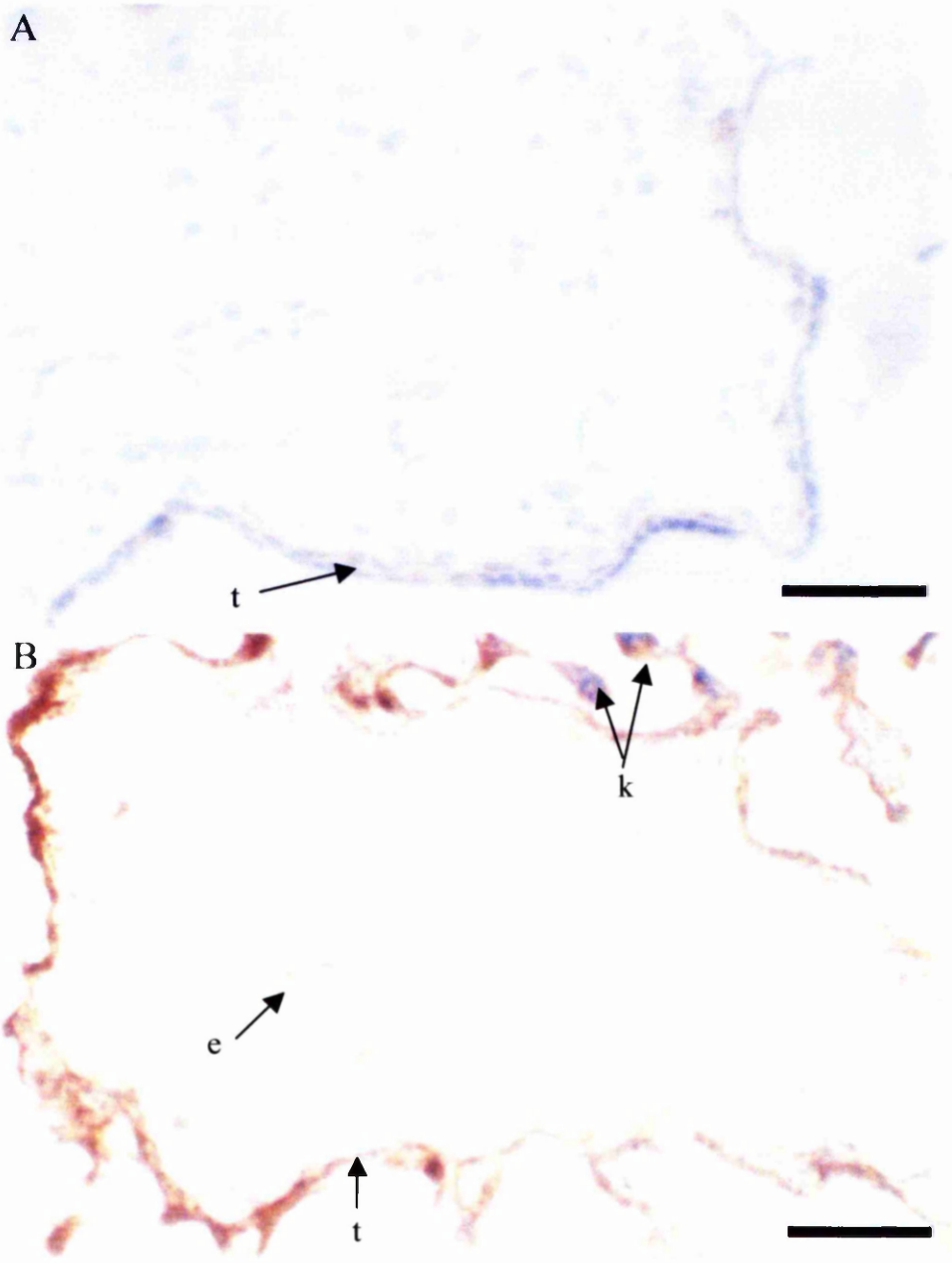


Figure 3.1.3.1a *Immunohistochemical localisation of inhibin α-subunits in mid-trimester control (A) and DS (B) placental sections. k=syncytial knot, e=endothelium, t=trophoblast. Size bar in A=0.05mm, and in B=0.1mm.*

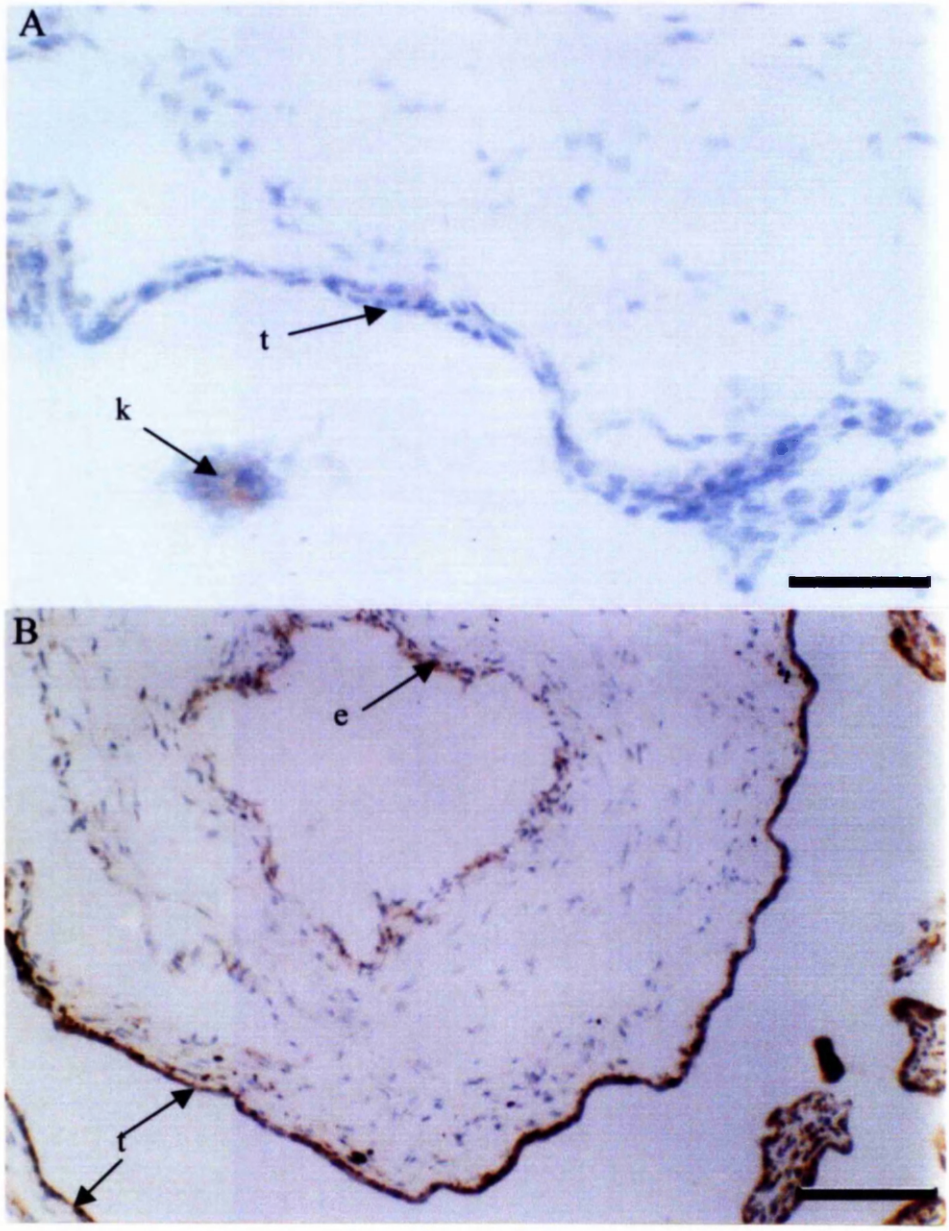


Figure 3.1.3.1b *Immunohistochemical localisation of inhibin/activin β_A-subunits in mid-trimester control (A) and DS (B) placental sections. k=syncytial knot, e=endothelium, t=trophoblast. Size bar in A=0.05mm, and in B=0.1mm.*

Figure 3.1.3.1c

Negative control section (primary antibody was replaced with control immunoglobulin fraction) Size bar=0.1mm.

	Alpha Subunit		Beta A Subunit	
Gestation (weeks)	**Trophoblast**	**Stroma**	**Trophoblast**	**Stroma**
14	+ve	+ve	+ve	+ve
14	+ve	+ve	+ve	+ve
14	+ve	+ve	tie	tie
15	+ve	+ve	tie	tie
16	+ve	+ve	+ve	+ve
16	tie	-ve	tie	+ve
16	-ve	tie	+ve	+ve
17	tie	tie	tie	+ve
17	tie	tie	+ve	+ve
17	+ve	+ve	+ve	tie
17	+ve	tie	+ve	tie
18	tie	+ve	tie	-ve
18	+ve	tie	tie	-ve
18	+ve	tie	+ve	+ve
18	+ve	-ve	tie	tie
19	tie	tie	+ve	-ve
19	-ve	+ve	tie	tie
19	-ve	-ve	-ve	+ve
19	tie	-ve	tie	tie
20	tie	tie	tie	+ve
Total -ve	3	4	1	3
Total ties	7	8	10	7
Total +ve	10	8	9	10
p value from sign	0.0923	0.3877	0.0215	0.0923

Table 3.1.3.2 *Ties and positive and negative differences shown in staining intensity of each matched pair of slides for staining with both subunits. Total number of pairs falling into each category is summarised at the foot of the table, with the p value as determined by the sign test, also shown.*

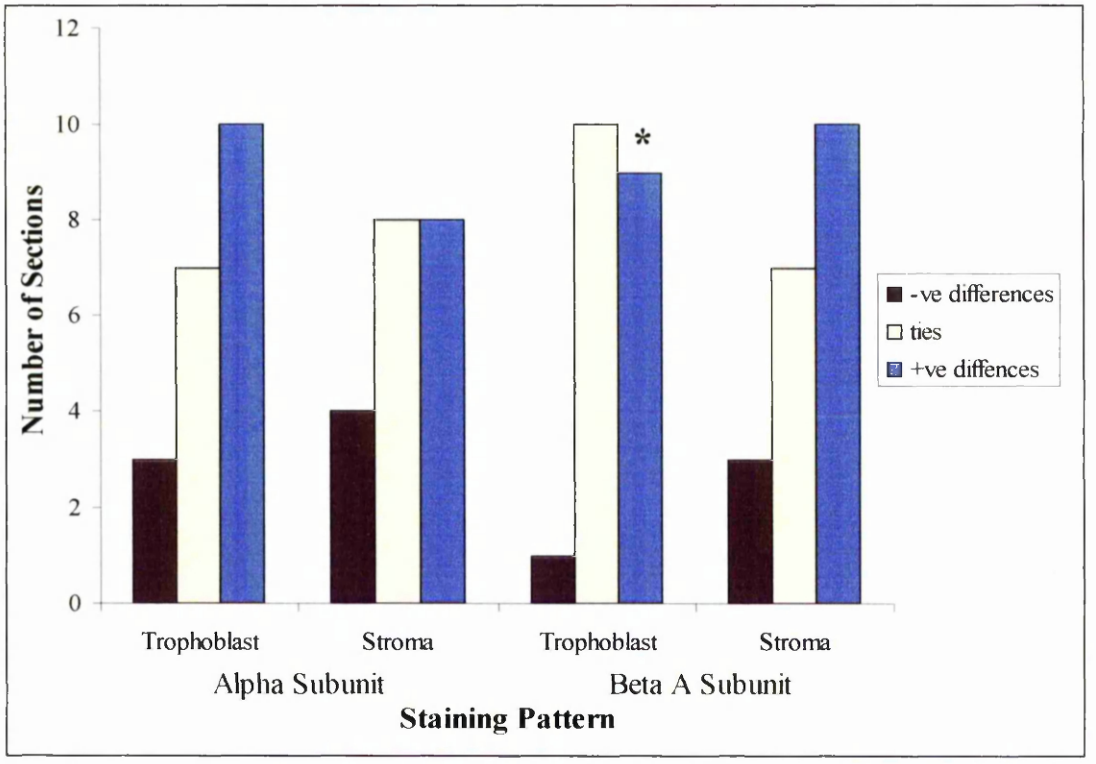


Figure 3.1.3.2 *Bar chart of negative differences, positive differences and ties in inhibin α and β_A subunit staining intensity in paired tissue sections. * $p<0.05$ according to sign test.*

3.1.4 Epidermal Growth Factor (EGF) Levels

3.1.4.1 Placenta

A preliminary study of the concentration of EGF in placental samples indicated that placental levels of this growth factor reflected only the amount of blood remaining in the placental extract. This was noted due to the large range of concentrations between samples and also because samples that were particularly blood stained had the highest concentration of EGF. Consequently, placental levels could not be further studied due to the confounding effects of contaminating blood.

3.1.4.2 Maternal Serum

Regressed median control levels of EGF in maternal serum, expressed as pg/ml, were calculated using a quadratic curve fit on non transformed data resulting in the equation $y=30641.263333-3680.136667x+113.833333x^2$ (figure 3.1.4.2a). Actual and regressed control median values are outlined in table 3.1.4.2. All results were converted to MoMs

using the regressed median value at the appropriate gestation. Median levels of EGF in maternal serum from DS pregnancies did not differ significantly from the control group. Control median MoM was 0.968 while the median in DS pregnancies was 1.026MoM ($p=0.639$). Individual cases are illustrated in figure 3.1.4.2b. Maternal serum levels of EGF were compared with 10 non-pregnant serum samples (5 female and 5 male). Median pregnancy levels were found to be significantly higher (958.3pg/ml versus 382.5 pg/ml, $p<0.001$).

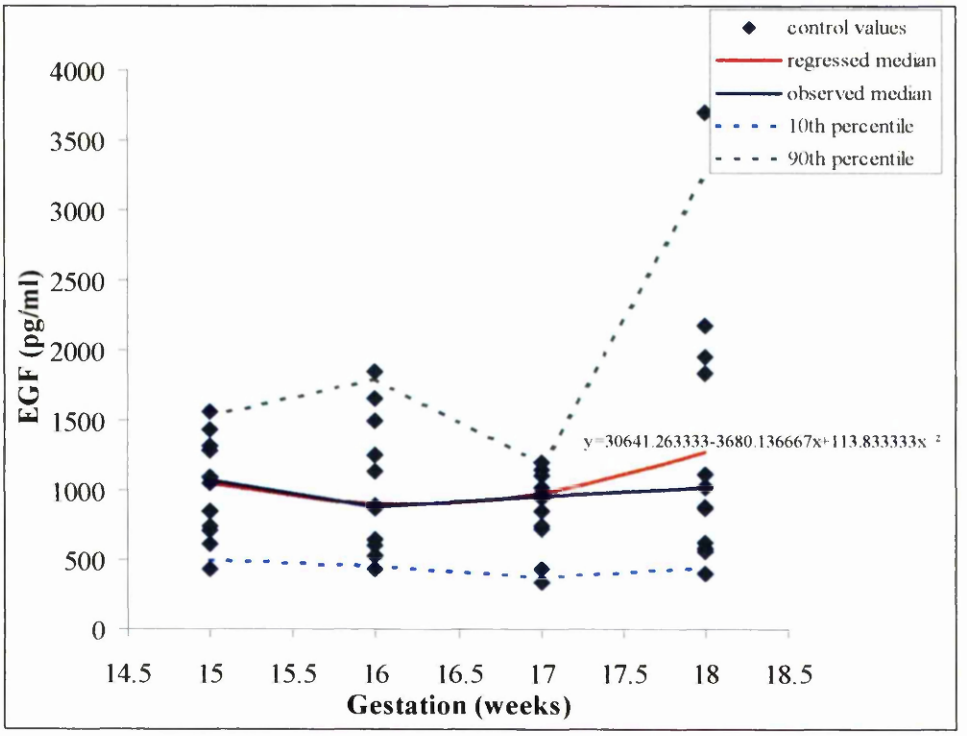


Figure 3.1.4.2a *Individual maternal serum EGF control data points (pg/ml), with actual and regressed median and 10th and 90th percentile lines plotted.*

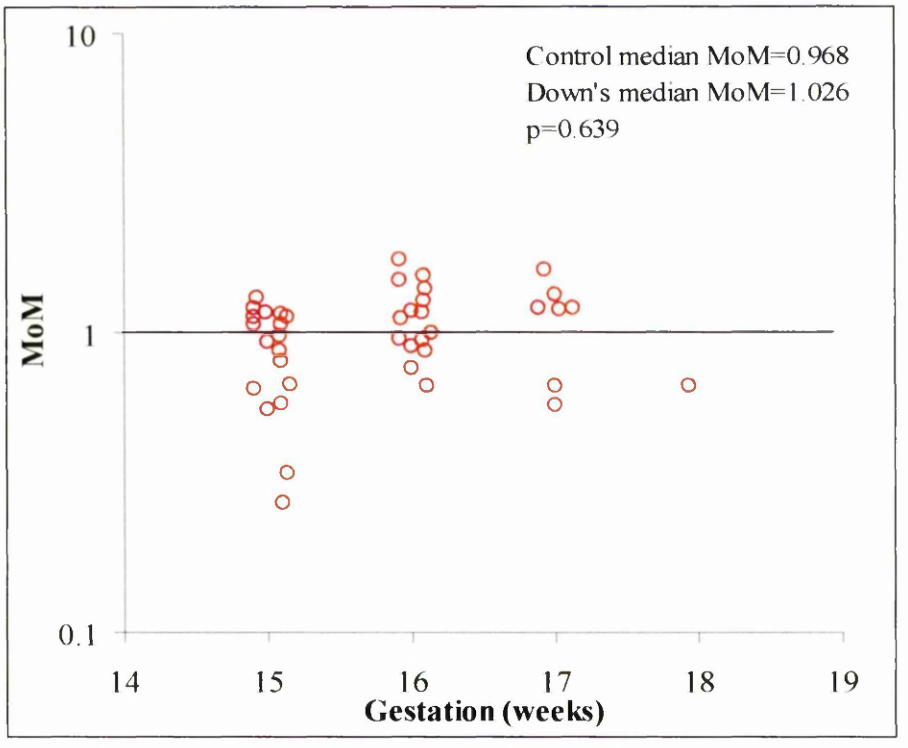


Figure 3.1.4.2b *DS maternal serum EGF MoMs.*

Gestation	EGF Medians (pg/ml)		$TGF\beta_1$ Medians (pg/ml)	
(weeks)	Regressed	Observed	Regressed	Observed
15	1051.71	1072.1	22894	30565
16	900.41	882.7	14081	15370
17	976.77	958.3	12397	11240
18	1280.80	1020.2	15625	12410

Table 3.1.4.2 *Observed and regressed control serum EGF and $TGF\beta_1$ levels at each week of gestation. Results are expressed as pg/ml.*

3.1.4.3 Maternal Urine

Regressed median control levels of EGF in maternal urine, expressed as pg/mg creatinine, were calculated using an inverse curve fit on $\log_{10}$ transformed data resulting in the equation y=4.693877+(1.717917÷x) (figure 3.1.4.3a). Actual and regressed control median values are outlined in table 3.1.4.3. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 0.932, while the DS median MoM was significantly reduced to 0.726MoM (p=0.011). Individual cases are illustrated in figure 3.1.4.3b.

Gestation (weeks)	EGF Medians (pg/mg creatinine)	
	Regressed	Observed
10	73395.6	39050.1
11	70802.7	108916.2
12	68713.2	41035.4
13	66991.5	76402.2
14	65552.6	54246.9
15	64329.5	68113.5
16	63277.6	80188.6
17	62363.4	57883.8
18	61563.0	46146.2
19	60854.1	65952.1
20	60224.0	56206.8
21	59659.5	75442.8

Table 3.1.4.3 *Observed and regressed control maternal urine EGF at each week of gestation. Results are expressed as pg/mg creatinine.*

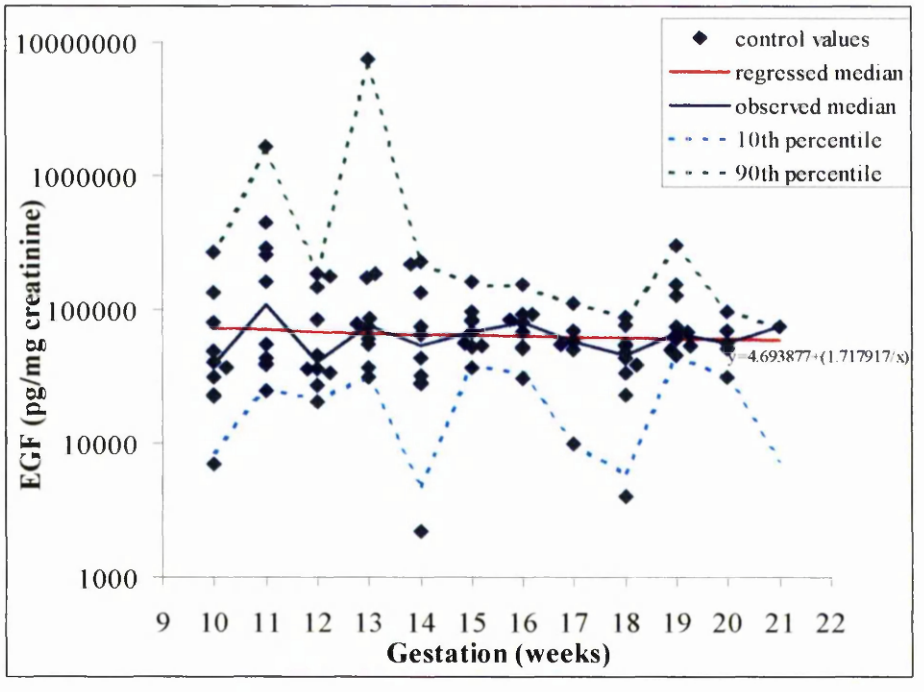


Figure 3.1.4.3a *Individual maternal urine EGF control data points (log_{10} pg/mg creatinine), with actual and regressed median and 10^{th} and 90^{th} percentile lines plotted.*

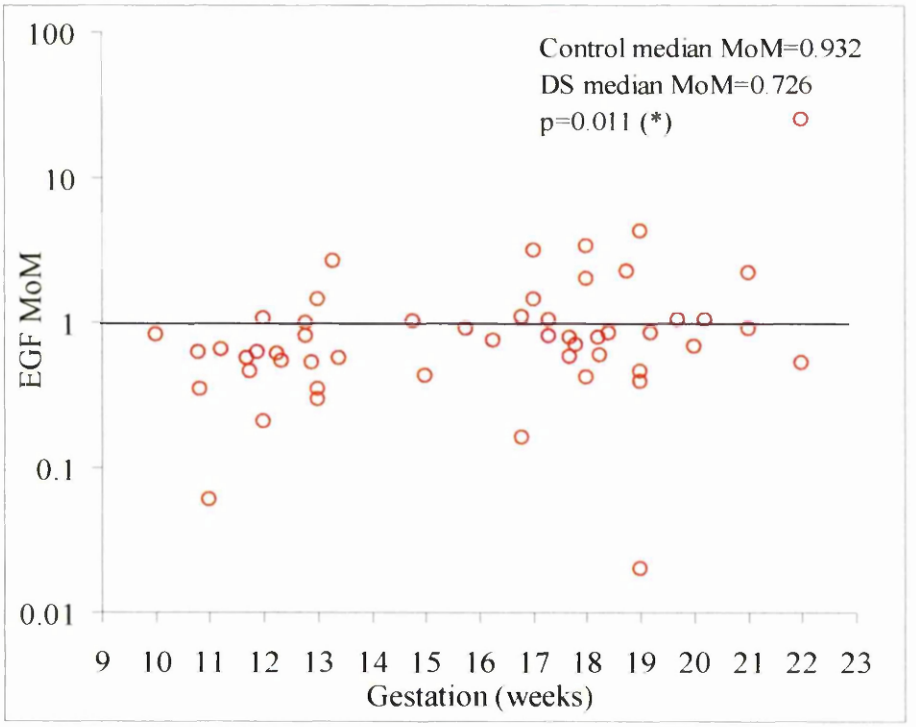


Figure 3.1.4.3b *DS maternal urine EGF MoMs.*

3.1.4.4 Amniotic Fluid

Regressed median control levels of EGF in amniotic fluid, expressed as pg/ml, were calculated using an inverse curve fit on non transformed data resulting in the equation y=10.743525-(88.036156÷x) (figure 3.1.4.4a). Actual and regressed control median values are outlined in table 3.1.4.4. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 0.991, while the DS median MoM was significantly reduced to 0.499MoM ($p<0.0001$). Individual cases are illustrated in figure 3.1.4.4b.

Gestation	EGF Medians (pg/ml)		$TGF\beta_1$ Medians (pg/ml)	
(weeks)	Regressed	Observed	Regressed	Observed
15	4.874	4.621	432.707	391.80
16	5.241	4.137	403.586	390.65
17	5.565	5.349	376.425	480.65
18	5.823	7.321	351.091	424.85
19	6.110	4.646	327.463	335.30
20	6.341	7.350	305.425	180.47

Table 3.1.4.4 *Observed and regressed control amniotic fluid EGF and $TGF\beta_1$ levels at each week of gestation. Results are expressed as pg/ml.*

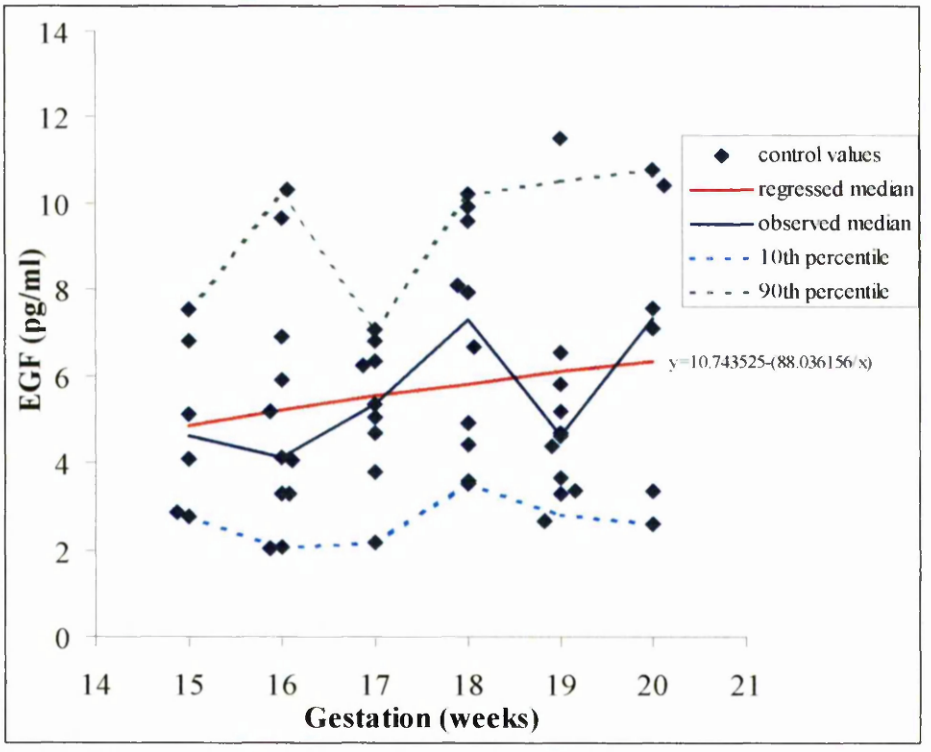


Figure 3.1.4.4a *Individual amniotic fluid EGF control data points (pg/ml), with actual and regressed median and 10^{th} and 90^{th} percentile lines plotted.*

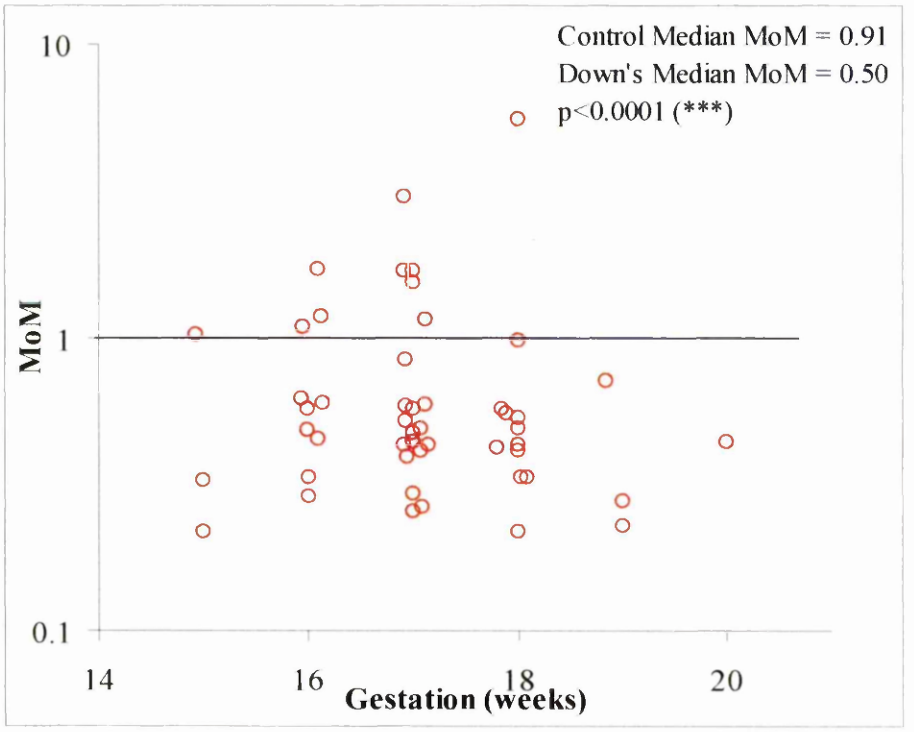


Figure 3.1.4.4b *DS amniotic fluid EGF MoMs.*

3.1.5 Transforming Growth Factor Beta 1 (TGFβ_1) Levels

3.1.5.1 Placenta

Regressed median control levels of TGFβ_1 in placental tissue, expressed as pg/μg protein were calculated using a quadratic curve fit on non-transformed data, resulting in the equation $y=10.309955-0.984298+0.028265x^2$ (figure 3.1.5.1a). Actual and regressed control median values are outlined in table 3.1.5.1. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 1.02, while the DS median MoM was significantly reduced to 0.675 ($p=0.002$). Individual cases are illustrated in figure 3.1.5.1b.

Gestation (weeks)	$TGF\beta_1$ Medians (pg/μg protein)	
	Regressed	Observed
14	2.070	2.089
15	1.905	1.550
16	1.797	2.139
17	1.745	1.731
18	1.750	1.616
19	1.812	1.560
20	1.930	2.254

Table 3.1.5.1 *Observed and regressed control placental $TGF\beta_1$ at each week of gestation. Results are expressed as pg/μg protein.*

3.1.5.2 Maternal Serum

Regressed median control levels of $TGF\beta_1$ in maternal serum, expressed as pg/ml, were calculated using a quadratic curve fit on $\log_{10}$ transformed data resulting in the equation $y=26.2215-2.6259x-0.779x^2$ (figure 3.1.5.2a). Actual and regressed control median values are outlined in table 3.1.5.2a. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median levels of $TGF\beta_1$ in maternal serum from DS pregnancies did not differ significantly from the control group. Control median MoM was 0.983 while in DS pregnancies it was 0.927MoM ($p=0.720$). Individual cases are illustrated in figure 3.1.5.2b. Maternal serum levels of $TGF\beta_1$ were compared with 10 non-pregnant serum samples (5 female and 5 male). No significant difference was found between groups in serum $TGF\beta_1$ concentrations ($p>0.05$).

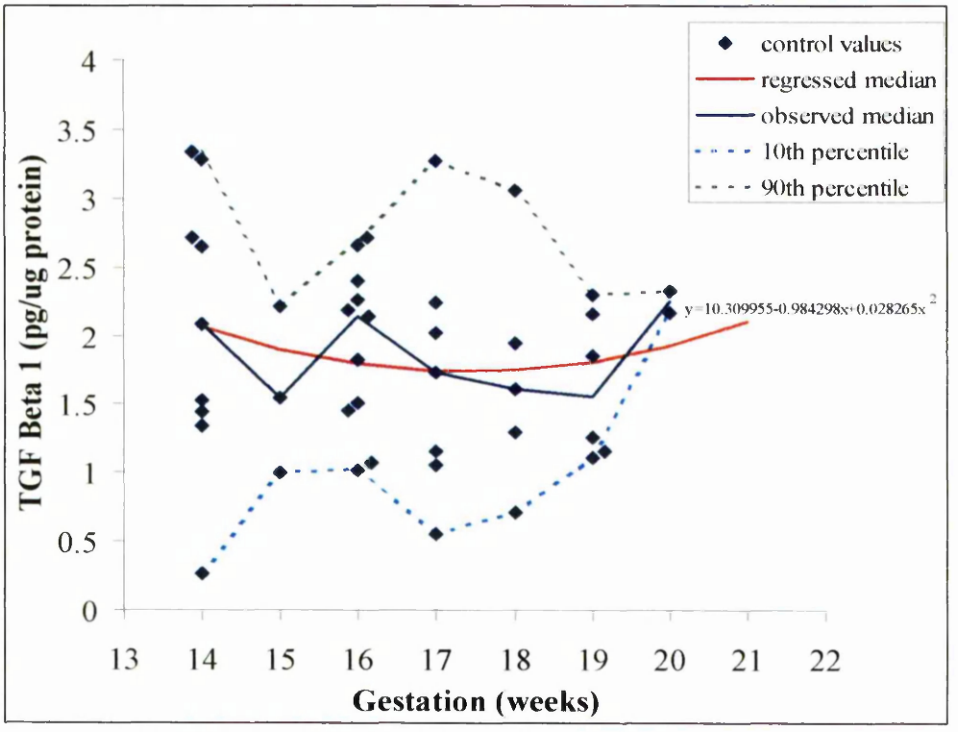


Figure 3.1.5.1a *Individual placental TGFβ_1 control data points (pg/μg protein), with actual and regressed median and 10th and 90th percentile lines plotted.*

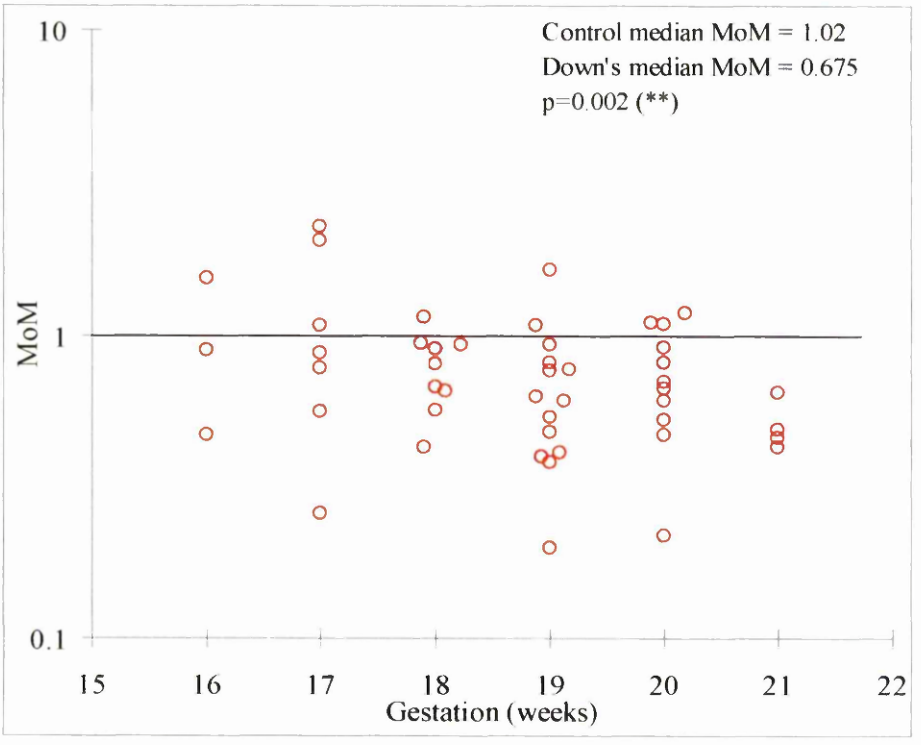


Figure 3.1.5.1b *DS placental TGFβ_1 MoMs.*

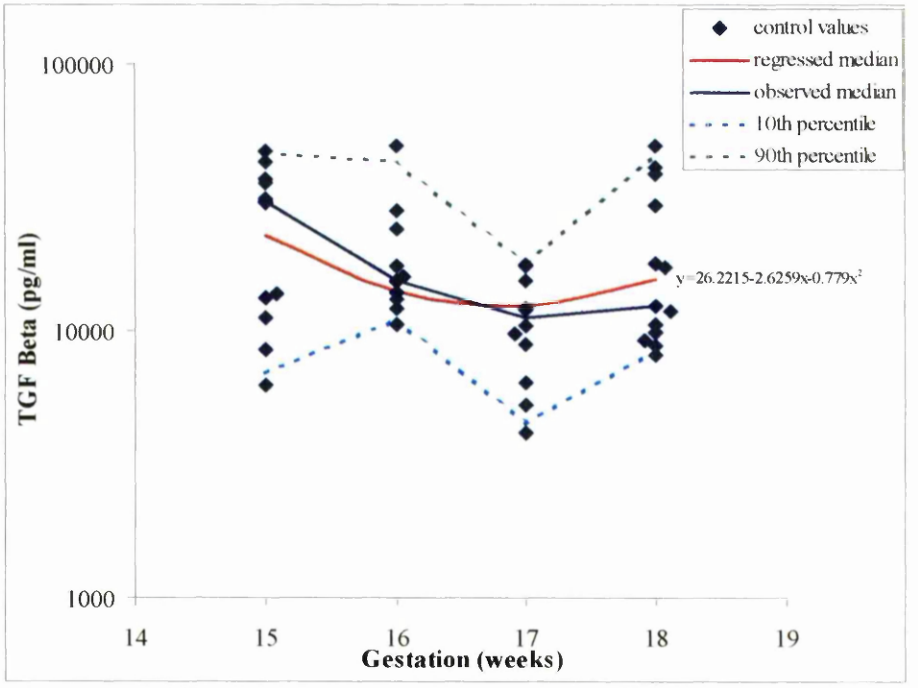


Figure 3.1.5.2a *Individual maternal serum TGF β_1 control data points (log_{10} pg/ml), with actual and regressed median and 10^{th} and 90^{th} percentile lines plotted.*

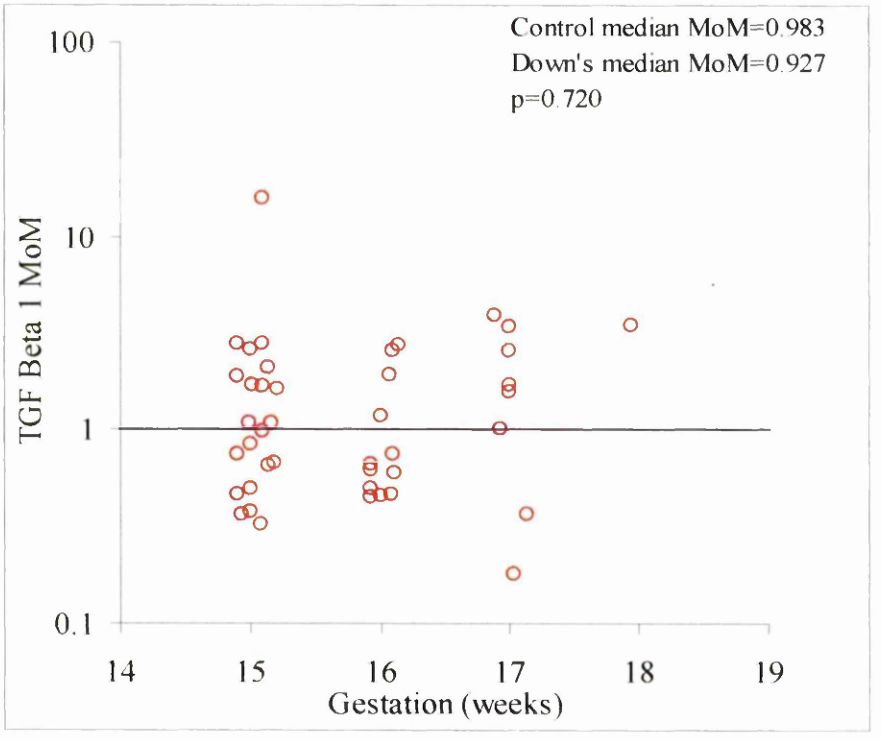


Figure 3.1.5.2b *DS maternal serum TGFβ_1 MoMs.*

3.1.5.3 *Maternal Urine*

Urinary levels of TGFβ_1 were below the limits of detection (15pg/ml) of the assay used.

3.1.5.4 *Amniotic Fluid*

Regressed median control levels of TGFβ_1 in amniotic fluid, expressed as pg/ml, were calculated using linear regression on $\log_{10}$ transformed data resulting in the equation y=3.090064-0.0302x (figure 3.1.5.4a). Actual and regressed control median values are outlined in table 3.1.5.4. All results were converted to MoMs using the regressed median value at the appropriate gestation. The median control MoM was 1.088, while the DS median MoM was significantly reduced to 0.685MoM (p=0.0064). Individual cases are illustrated in figure 3.1.5.4b.

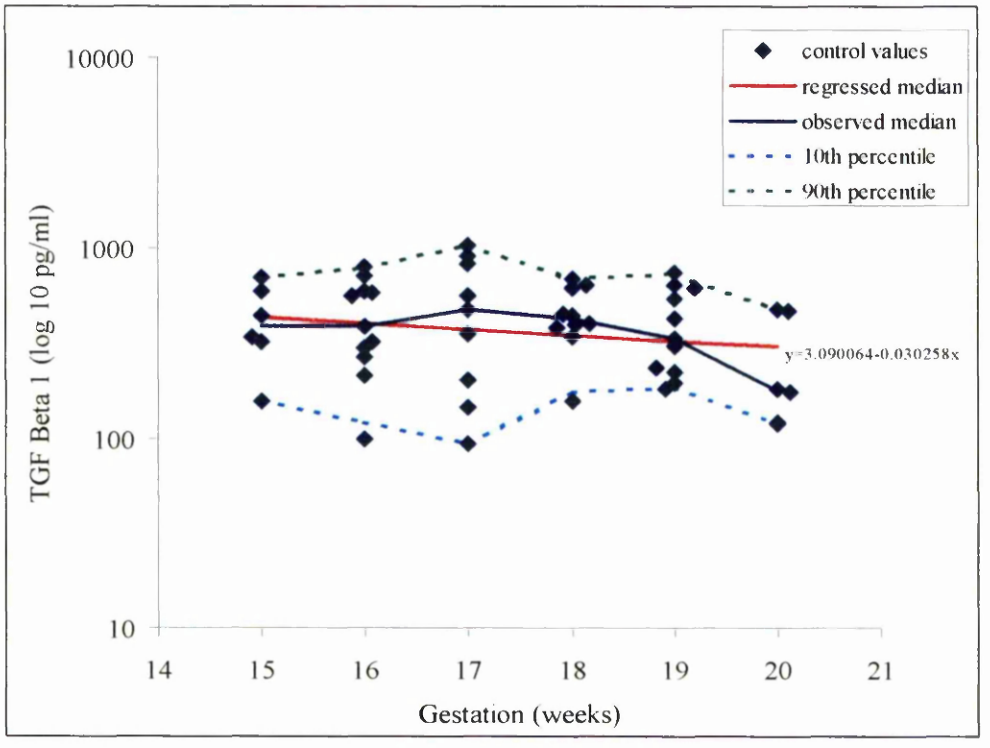


Figure 3.1.5.4a *Individual amniotic fluid TGF β_1 control data points ($\log_{10}$ pg/ml), with actual and regressed median and 10th and 90th percentile lines plotted.*

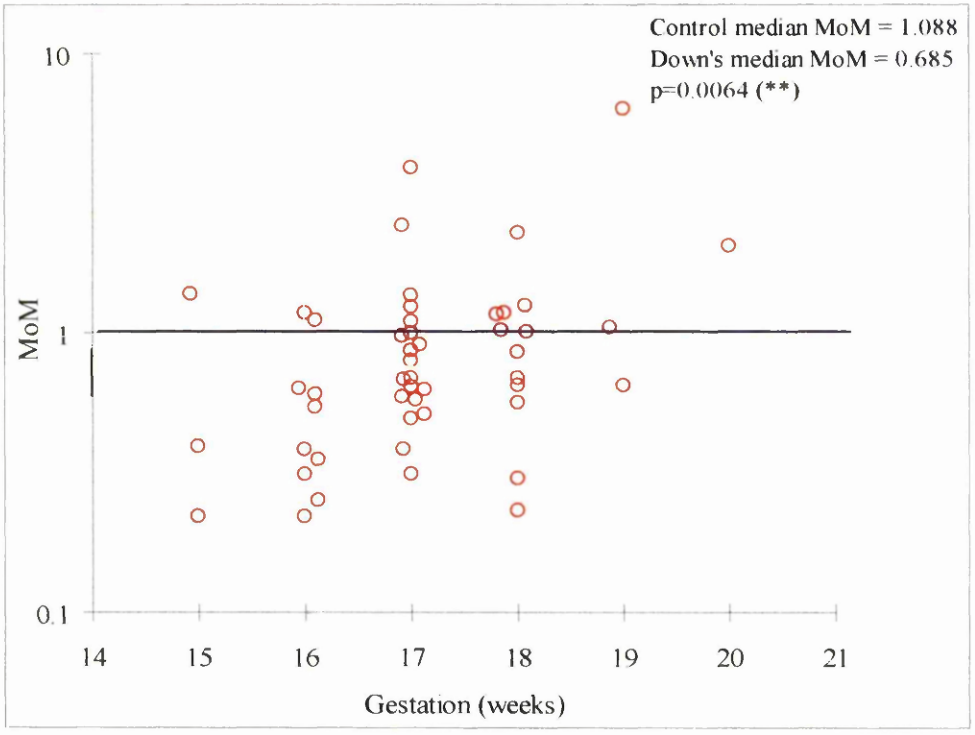


Figure 3.1.5.4b *DS amniotic fluid TGFβ_1 MoMs.*

3.1.6 Correlations Between Marker Levels

Correlations between marker levels were calculated using Pearson's Pairwise correlation coefficient (r). Previously determined fβ-hCG and ihCG levels in the placental and serum samples (Newby *et al* 1997) were used in these calculations.

3.1.6.1 Inhibin and activin

Correlations between serum and placental levels

Using Pearson's bivariate pairwise correlation, maternal serum and placental inhibin-A and activin-A MoM's were compared. Control placental inhibin-A and activin-A showed some correlation (r=0.4255, p=0.004). A weak association was found between the inhibin-A and activin-A MoMs in the DS placental samples with a correlation coefficient of 0.2878 (p=0.025). These correlations are illustrated in fig 3.1.6.1. This situation was mirrored in DS maternal serum, with inhibin-A and activin-A MoMs showing a degree of correlation (r=0.3055, p=0.05). No significant correlation was observed between serum and placental levels of inhibin-A or activin-A in DS pregnancies. This lack of correlation between placental and maternal serum levels of inhibin-A and activin-A was further explored by examining the levels in fifteen placental homogenates and corresponding serum samples collected at the time of termination. This eliminates the possibility that any change in analyte levels was due to

the difference in time from obtaining the serum-screening sample (around 15-17 weeks gestation), and the time of obtaining the placental sample at termination (approximately 2-3 weeks later). This produced a strong correlation between placental and serum levels of inhibin-A (r=0.7097, p=0.003), but not for activin-A (r=0.189, p=0.507). A summary of all correlation coefficients can be found in table 3.1.6.1a.

Correlations between hCG and inhibin-A/activin-A in placenta and maternal serum

Placental and maternal serum levels of inhibin-A and activin-A found in the present study were compared with those of total hCG and free beta hCG previously reported by Newby *et al* (1997). Control placental levels of inhibin-A and free beta hCG show a significant correlation (r=0.4122, p=0.006) with a similar, but not statistically significant trend being seen between inhibin-A and total hCG. There appears to be little association between activin-A levels and total or free beta hCG levels in control placental samples. In DS placental samples, no correlation could be found between either activin-A or inhibin-A levels and total or free beta hCG levels. There was a significant correlation between DS maternal serum activin-A and both total and free beta hCG (r=0.6619, p=0.014 and r=0.6421, p=0.018 respectively). There was, however, no significant correlation between inhibin-A and either total or free beta hCG. All correlation coefficients are summarised in table 3.1.6.1b.

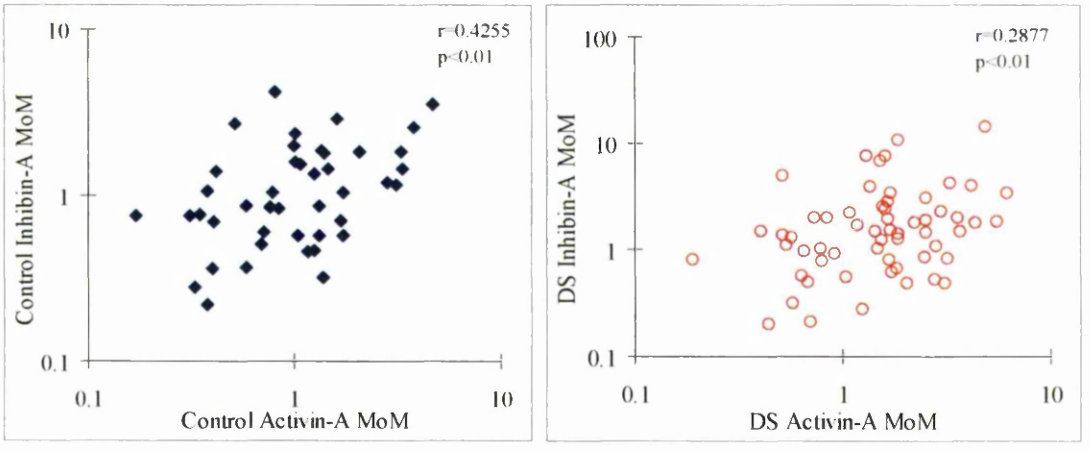


Figure 3.1.6.1 *Correlations between inhibin-A and activin-A in control (blue) and DS (red) placental samples. The correlation coefficient (r) and significance (p) is as calculated using Pearson's bivariate pairwise correlation.*

MoM values compared	DS Samples	Control Samples
Placental inh-A/act-A	r=0.2877, p=0.0025, (61)	r=0.4255, p=0.004, (43)
Placental/serum act-A	r=0.1982, p=0.220, (40)	/
Placental/serum inh-A	r=-0.2006, p=0.214, (40)	/
Serum inh-A/act-A	r=0.3055, p=0.05, (41)	/
Placental/pre-TOP serum act-A	r=0.1859, p=0.507 (15)	/
Placental/pre-TOP serum inh-A	r=0.7097, p=0.003 (15)	/

Table 3.1.6.1a *Summary of correlation coefficients (r) obtained using Pearson's bivariate pairwise correlation on inhibin-A (inh-A) and activin-A (act-A) MoM values. The number of samples on which the correlation was carried out is given in brackets, while the significance of the correlation is given as the p value.*

MoMs compared	Control Placental	DS placental	DS serum
Inh-A/ fβ-hCG	r=0.4122	r=-0.0581	r=0.1221
	p=0.006 (43)	p=0.756 (31)	p=0.694 (13)
Inh-A/ihCG	r=0.2469	r=0.2848	r=0.3594
	p=0.110 (43)	p=0.120 (31)	p=0.228 (13)
Act-A/ fβ-hCG	r=-0.1005	r=-0.1357	r=0.6421
	p=0.522 (43)	p=0.467 (31)	p=0.018 (13)
Act-A/ihCG	r=0.0831	r=-0.0108	r=0.6619
	p=0.596 (43)	p=0.954 (31)	p=0.014 (13)

Table 3.1.6.1b *Summary of correlation coefficients (r) obtained using Pearson's bivariate pairwise correlation on inh-A and act-A MoM values compared with previously reported hCG MoMs. The number of samples on which the correlation was carried out is given in brackets, while the significance of the correlation is given as the p value.*

3.1.6.2 Growth Factors

Using Pearson's bivariate pairwise correlation, amniotic fluid levels of EGF and $TGF\beta_1$ expressed as MoM, were compared to fβ-hCG and ihCG levels previously reported (Newby *et al* 1997). Little or no correlation was observed between the growth factor levels and both forms of hCG (table 3.1.6.2). Correlation coefficients between DS

placental and amniotic fluid $TGF\beta_1$ levels were also calculated in the same way. There was no significant association between these values r=0.031, p=0.853, n=39.

MoMs Compared	Amniotic Fluid
fβ-hCG /EGF	r=0.070 p=0.813 n=14
ihCG/EGF	r=-0.259 p=0.370 n=14
EGF/$TGF\beta_1$	r=-0.087 p=0.548 n=50
fβ-hCG /$TGF\beta_1$	r=0.319 p=0.266 n=14
ihCG/$TGF\beta_1$	r=0.363 p=0.202 n=14

Table 3.1.6.2 *Summary of correlation coefficients (r) obtained using Pearson's bivariate pairwise correlation on DS amniotic fluid EGF and $TGF\beta_1$ MoM values compared with previously reported hCG MoMs. The number of samples on which the correlation was carried out is indicated by the n value, while the significance of the correlation is given as the p value.*

3.2 Development of ELISA Based hCG β mRNA Quantification Method

3.2.1 Specificity of Assay

Before the hCG mRNA assay could be used to measure the quantity of mRNA present in test samples, the specificity of the assay had to be determined. This was firstly assessed using one of the probes, and a complementary probe as a primer pair for PCR (see section 2.5.2.1 for sequences). The specificity in assay format was confirmed by using samples known to contain no hCG mRNA and also by substituting one of the probes for the complementary probe which would only bind to DNA.

3.2.1.1 Confirmation of Probe Specificity by PCR

Three different placental RNA samples were reverse transcribed and amplified by PCR following the method outlined in section 2.5.2.2. Figure 3.2.1.1a illustrates that a fragment of the correct size (539bp) was obtained in all samples. To further confirm the identity of the amplified region, AluI restriction digest was carried out, which was

expected to produce bands of 345bp and 194bp. As can be seen from figure 3.2.1.1b bands of the correct size were obtained.

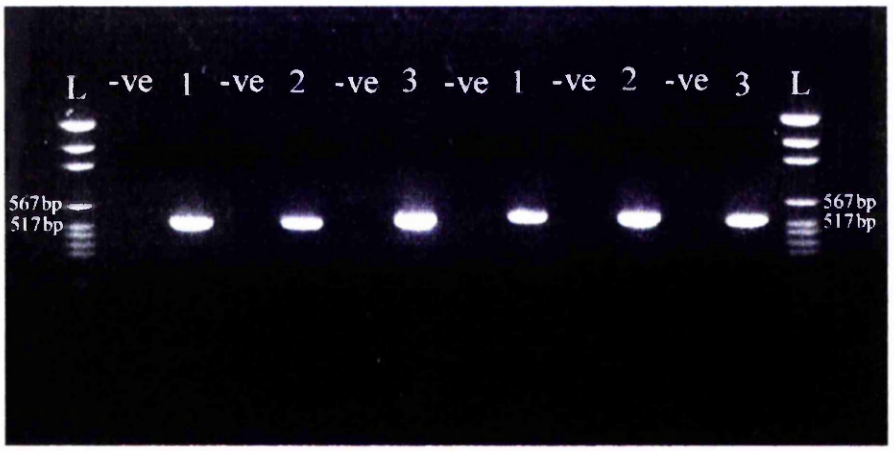


Figure 3.2.1.1a *Results of PCR amplification of three different placental cDNA samples (1,2&3). A no DNA negative control sample (-ve) was loaded beside each PCR reaction sample along with a DNA marker (L). A band of 539bp is clearly visible for each reaction.*

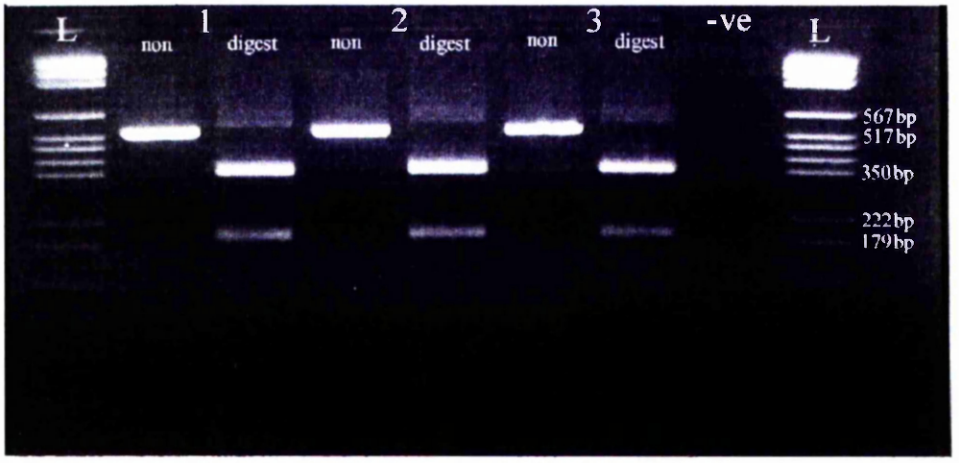


Figure 3.2.1.1b *Results of AluI digest of PCR product from three different placental samples (1,2&3). An undigested control (non) and digested (digest) sample for each placenta was loaded, along with a no DNA PCR negative control (-ve) and a DNA marker (L). A band of 539bp is clearly visible for each control reaction and two bands of 345bp and 194bp are visible for each digested sample.*

3.2.1.2 Confirmation of Specificity in Assay Format

Total RNA isolated from cultured fibroblast cells, which do not express hCG mRNA, was assayed using this method and produced a result close to zero. Substituting the biotin labelled probe with the complementary probe, which would only specifically bind to any contaminating DNA, gave a result close to zero. Together these experiments exclude the possibility that the hCG probes bind to other mRNA sequences or that the assay system gives spurious results because of non-specific binding of either probes or antibody at any step in the procedure.

3.2.2 Reproducibility and Accuracy of Assay

To check the reproducibility of the assay, two samples were used as controls each time an assay was performed to determine the inter assay variation. Samples were also run repeatedly on one plate to give the intra assay coefficient of variation (CV). One sample with an average concentration of 4.47 arbitrary units was assayed in 9 wells of one plate giving a CV of 13.3% and another sample was assayed in 23 wells giving an average concentration of 5.51 units with a CV of 17%. For research purposes, a sample was re-assayed if the intra assay CV was >20%. In most cases, a good linear standard curve was obtained from the arbitrary set of placental RNA standards. Figure 3.2.2 illustrates a typical standard curve.

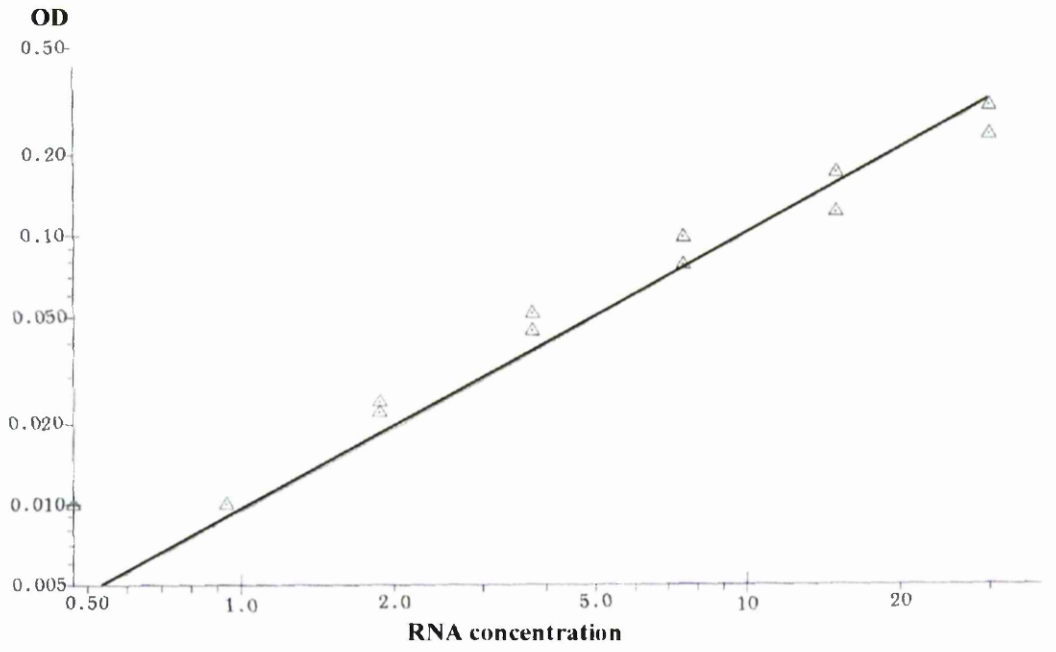


Figure 3.2.2 *Typical hCG RNA standard curve generated using Genesis software package.*

3.2.3 Comparison with Northern Blotting

A northern blot was carried out using various quantities of a total placental RNA sample per lane (1, 2, 5, 10 or 20μg) to assess the sensitivity of this method in comparison to the RNA assay (fig 3.2.3). As can be seen, it was difficult to pick up anything less than a 5-fold difference in target mRNA concentration at the lower concentrations, and a 2-fold difference at higher concentrations. As well as being less sensitive at determining differences between samples, more RNA is required to perform northern blots than is used in the plate based assay. From this blot using 10μg RNA per lane would be the minimum to allow accurate comparison of results.

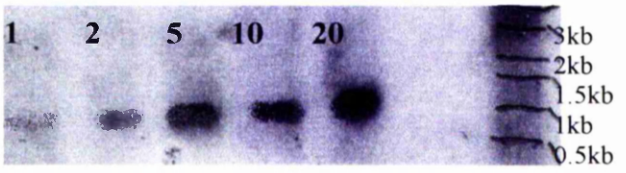


Figure 3.2.3 *Autoradiograph of hCG northern blot using varying quantities of RNA (indicated above each lane in μg). RNA size marker is on right hand side of figure, with sizes of each band indicated in kb.*

3.2.4 Comparison with Taq Man® Real Time PCR

Typical amplification plots for hCG and PPIA mRNA are illustrated in fig 3.2.4a and 3.2.4b respectively. The number of PCR cycles (x-axis) is plotted against the ΔRn value, which is an index of the quantity of PCR product (y-axis) for each individual reaction. This allows the rate of the PCR reaction for each sample to be visualised. These plots are used to set the threshold level from which the Ct value for each sample can be calculated. The threshold value should be within the logarithmic phase of the reaction and the negative control sample should have a Ct close to 40 at this threshold value. This method requires by far the least amount of starting total RNA (2μg is sufficient for several PCR reactions). Each batch of reactions can determine the quantity of both hCG and PPIA mRNA in 45 test samples, making this an extremely quick method of study a large quantity of samples.

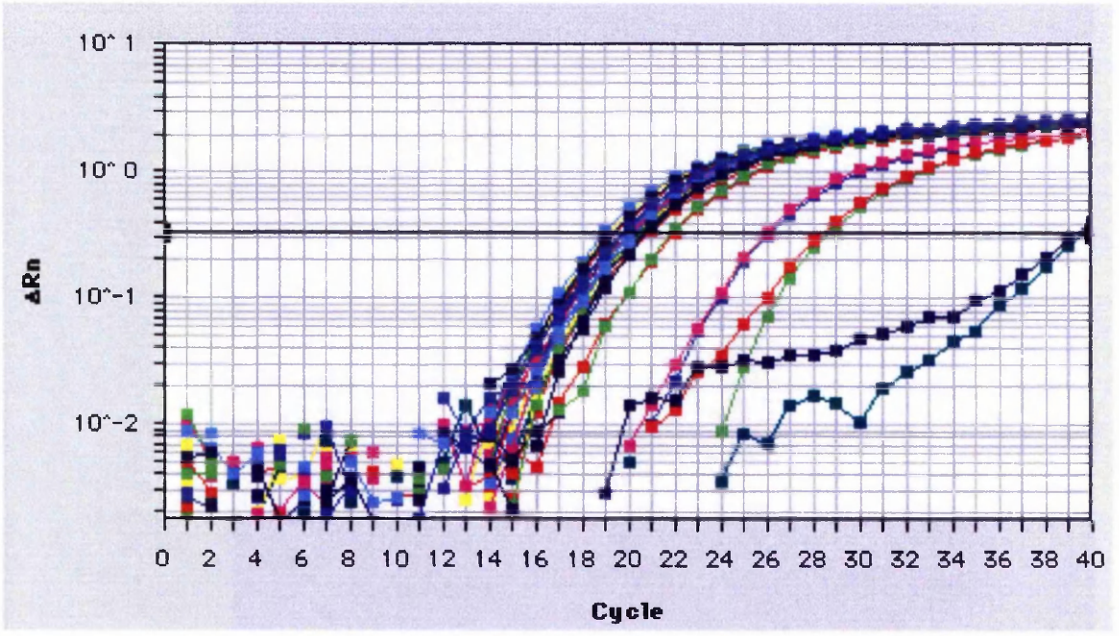


Figure 3.2.4a *Typical hCG mRNA real time PCR amplification plot. The threshold value (indicated by a black horizontal line) was set so that the PCR reactions had not yet reached a plateau and the negative control sample (dark blue and dark green lines) gave a Ct number close to 40. Amplification plots are of 24h-96h mRNA isolated from cultured trophoblast cells. 24h samples have very little hCG mRNA as indicated by the high Ct (magenta, blue, red and green lines).*

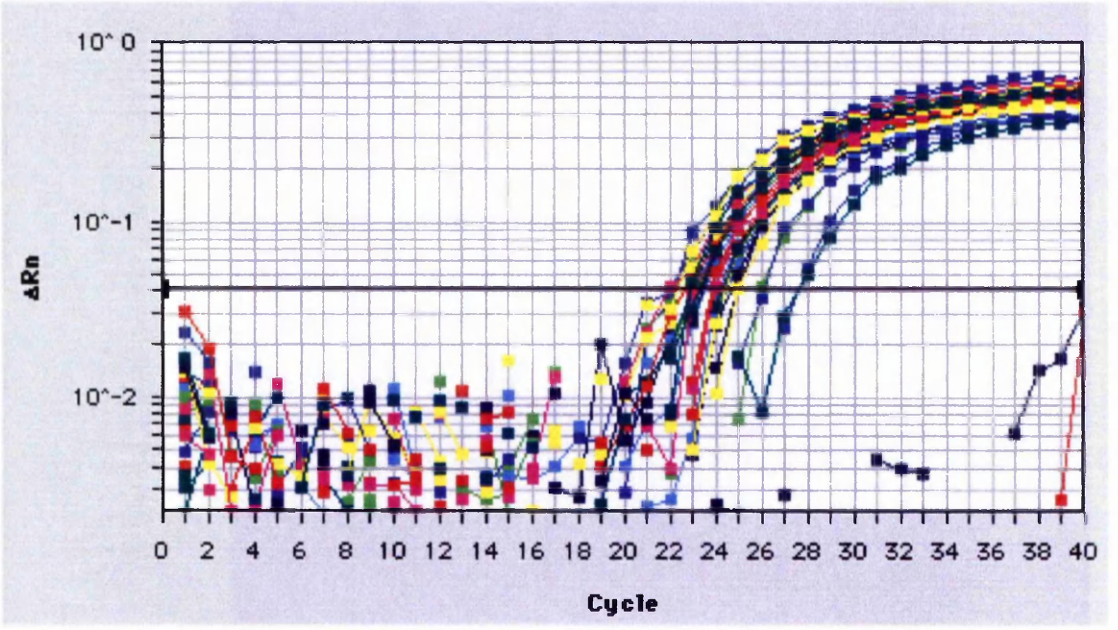


Figure 3.2.4b *Typical PPIA mRNA real time PCR amplification plot. The threshold value (indicated by a black horizontal line) was set so that the PCR reactions had not yet reached a plateau and the negative control sample (dark blue and red lines) gave a Ct number close to 40. Amplification lots are of 24h-96h mRNA isolated from cultured trophoblast cells.*

3.2.5 Correlation Between hCG mRNA Assay and Real Time PCR Methods

A selection of 62 RNA samples collected for the IFNα study, the results of which are out outlined in section 3.4, were assayed using both the plate based hCG mRNA assay and the real time PCR method to compare the results each gave. The $2^{-\Delta\Delta Ct}$ values obtained from the real time PCR method were compared to the hCG results divided by the GAPDH results obtained from the plate based method using the Pearson pairwise correlation. A significant correlation was observed between both data sets ($r=0.384$, $p=0.002$). These results are illustrated in fig 3.2.5.

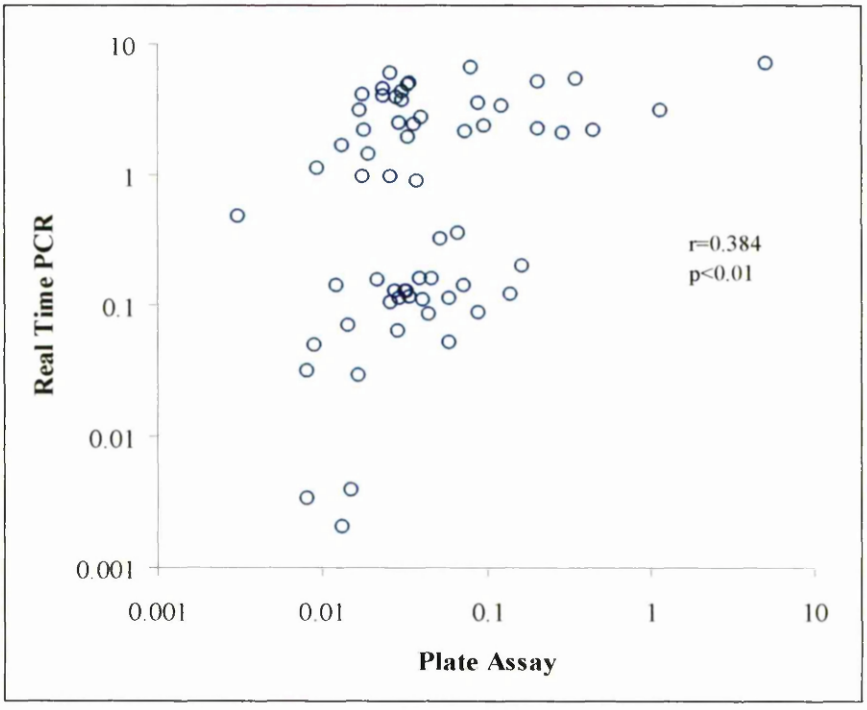


Figure 3.2.5 *Correlation between Real time PCR and plate based assay method of mRNA quantification. The correlation coefficient (r) and significance (p) is as calculated using Pearson's bivariate pairwise correlation*

3.3 Development and Characterisation of Cell Culture Method

3.3.1 Validation of Initial Cultures and Effects of Cryopreservation

As an initial step in developing the trophoblast culture method the viability and purity of cells was assessed both before and after cryopreservation.

3.3.1.1 Morphology and Purity

Purity of pre and post cryopreservation cells was gauged by carrying out immunocytochemistry using vimentin V9 and cytokeratin 5/6/18 antibodies on fixed trophoblasts after 24h in culture. Cultures were generally >90% pure. The differentiation of cytotrophoblasts to syncytiotrophoblast in fresh cultures was monitored at 24h intervals by light microscopy. After 24h the cells were mainly

mononuclear individual cells, and as the culture period progressed these cells fused into a continual syncytial layer (fig 3.3.1.1b).

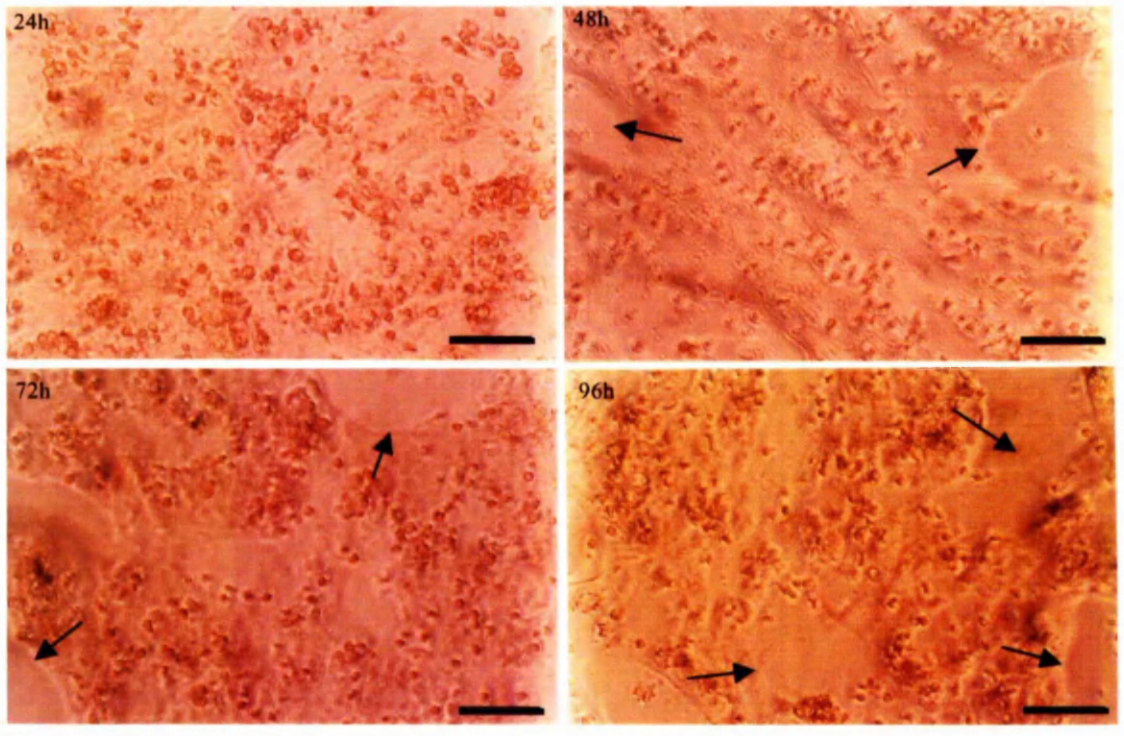


Figure 3.3.1.1b *Differentiation of trophoblasts over a 96h culture period. Photographs were taken at 24h intervals using phase contrast microscope. Arrows indicate areas with no cells from which trophoblasts have migrated during syncytialisation. Size bars represent 0.1mm.*

3.3.1.2 hCG and PLAP Secretion

Function and viability of the cells was determined by measuring hCG secretion and PLAP secretion from cytotrophoblasts as they syncytialised. Figure 3.3.1.2a shows the hCG results of 8 cultures, before and after freezing, taken as a ratio of total protein content of the cells in each well to account for variations in cell plating efficiency. As can be seen from this figure, very little hCG was produced in the first 24h of culture when the cytotrophoblasts were adhering to the culture surface. As the cells syncytialised the rate of hCG production increased to a peak at 72h, and then declined at 96h when most cells had formed syncytium. It is also clear that the cells did not behave normally after freezing, with the rate of hCG production significantly lower ($p=0.025$) at the 72h time point. This reduction in hCG production was accompanied by cells which did not adhere to the culture surface and did not appear to differentiate as they had done as fresh cells.

The rate of placental alkaline phosphatase (PLAP) secretion was at a peak at 24h, declined at 48h and then increased through the culture period (fig 3.3.1.2b) as the cells syncytialised. The peak at 24h is probably due to release of PLAP by syncytial fragments and other pieces of tissue, which are washed from the culture surface at the first medium change. In subsequent cultures the number of digests was decreased from 3 to 2, which gave cleaner preparations due to sharper percoll bands, thereby decreasing this peak of PLAP. There was no significant difference in the rate of PLAP secretion between fresh and frozen cultures.

3.3.1.3 Quantity of Cells

The effects of cryopreservation on the number of cells remaining in culture was investigated by measuring the total protein content and the DNA content of a cell lysate that had been scraped from duplicate wells of a 24 well culture dish at 24h time points. There was no significant difference in either the protein or DNA content in the wells at any time point when fresh cultures were compared to frozen cultures ($p>0.05$) using the Wilcoxon Matched pairs signed ranks test (fig 3.3.1.3a and fig 3.3.1.3b).

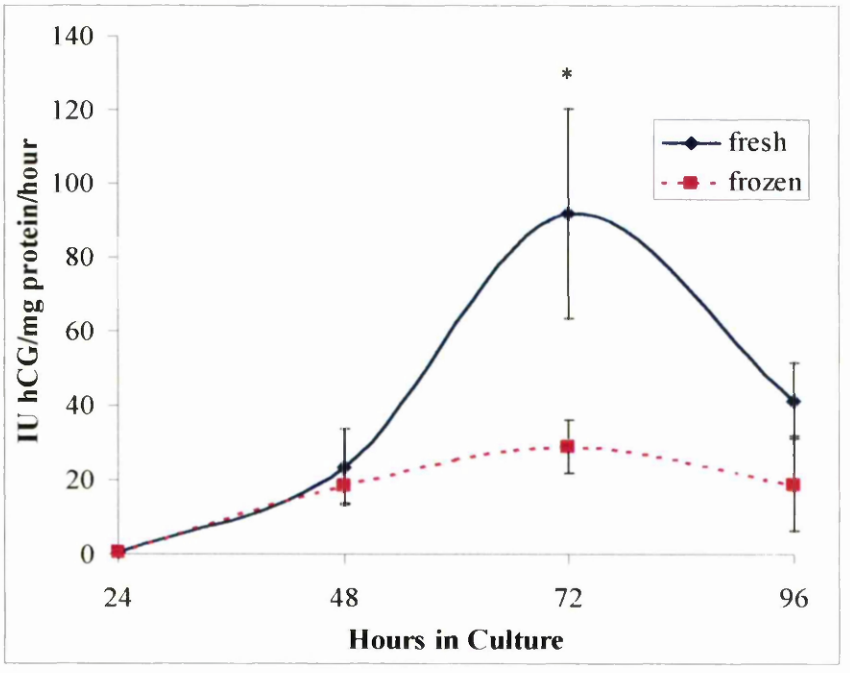


Figure 3.3.1.2a *Comparison of hCG secretion from trophoblast cultures before and after cryopreservation. Results are expressed a mean of 8 cultures ± sem in IU hCG per mg protein per hour. *$p<0.05$ by Wilcoxon Signed Ranks Test.*

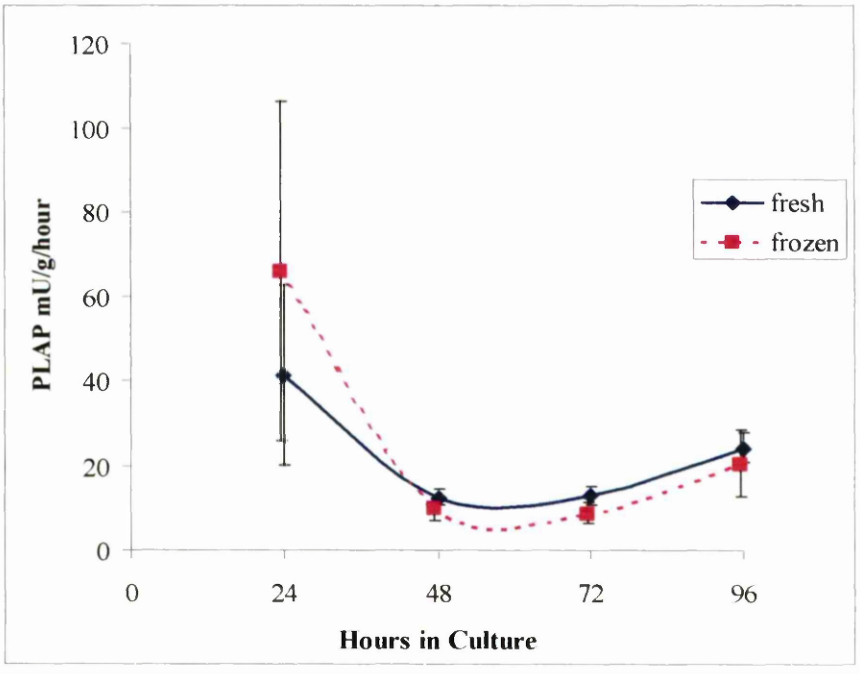


Figure 3.3.1.2b *Comparison of PLAP secretion from trophoblast cultures before and after cryopreservation. Results are expressed a mean of 8 cultures ± sem in mU PLAP per g protein per hour.*

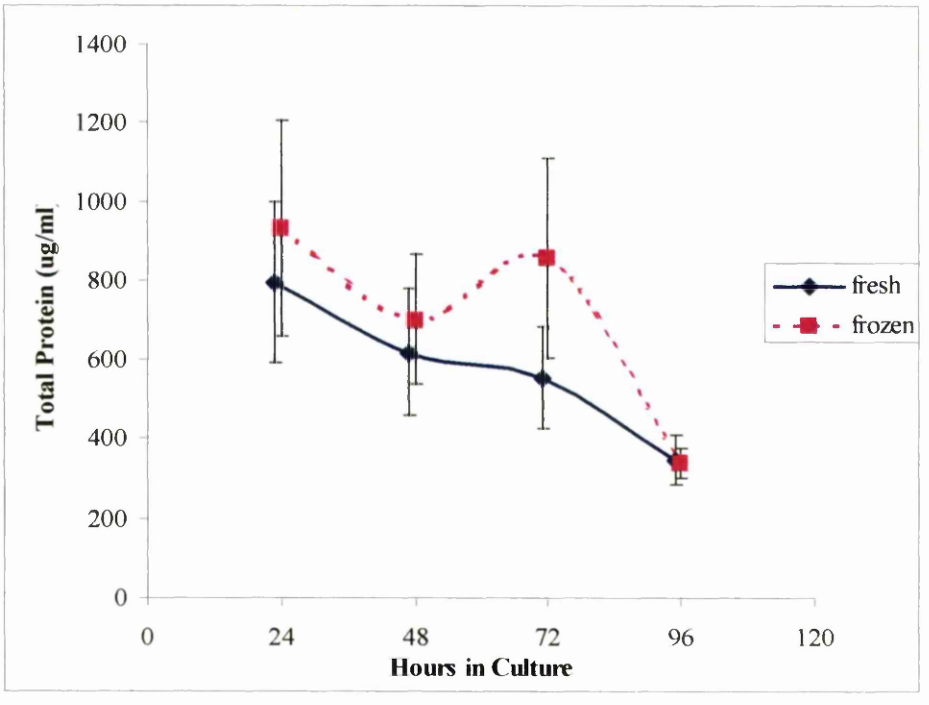


Figure 3.3.1.3a *Comparison of protein concentration in trophoblast cultures before and after cryopreservation. Results are expressed a mean of 7 cultures ± sem in µg protein per ml cell lysate.*

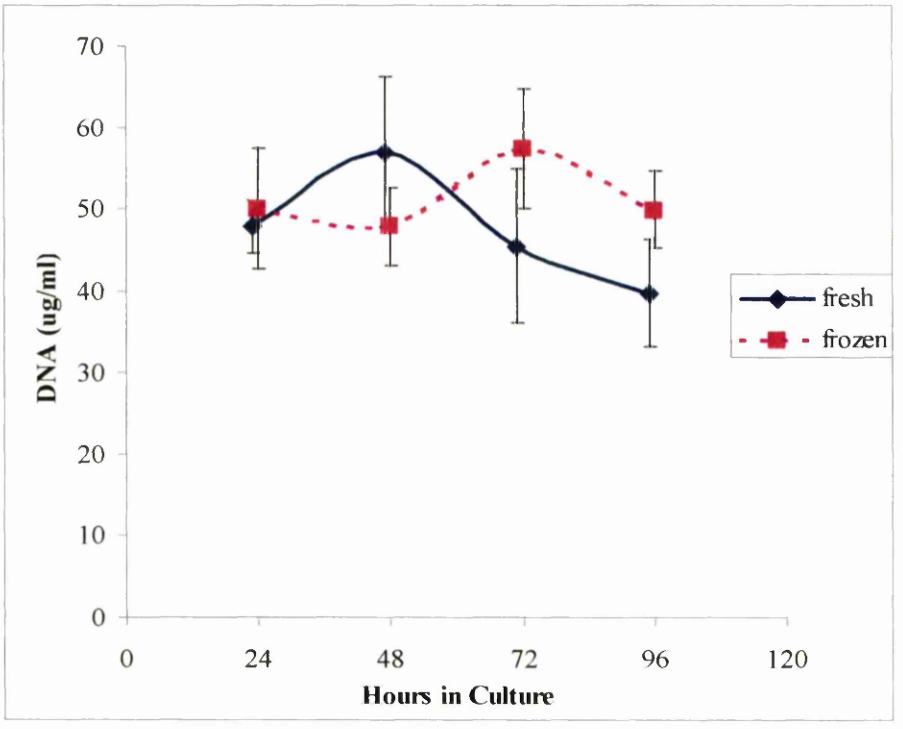


Figure 3.3.1.3b *Comparison of DNA concentration in trophoblast cultures before and after cryopreservation. Results are expressed a mean of 7 cultures ± sem in μg DNA per ml cell lysate.*

In addition to assessing the effect of cryopreservation on cell number using both methods, the accuracy of the DNA method and the protein method were assessed. The DNA and protein concentrations were compared using Pearson's pairwise correlation to determine if both methods of determining cell number were comparable. In both fresh and frozen cultures, a significant correlation was observed between both methods indicating that either would be an appropriate method of determining the number of cells present in culture well. For the fresh cultures $r=0.4495$ ($p=0.016$) and for the frozen cultures $r=0.4054$ ($p=0.032$). These results are illustrated in figure 3.3.1.3c. Time in culture had no significant effect on either protein or DNA content of cells ($p>0.05$, GLM Univariate).

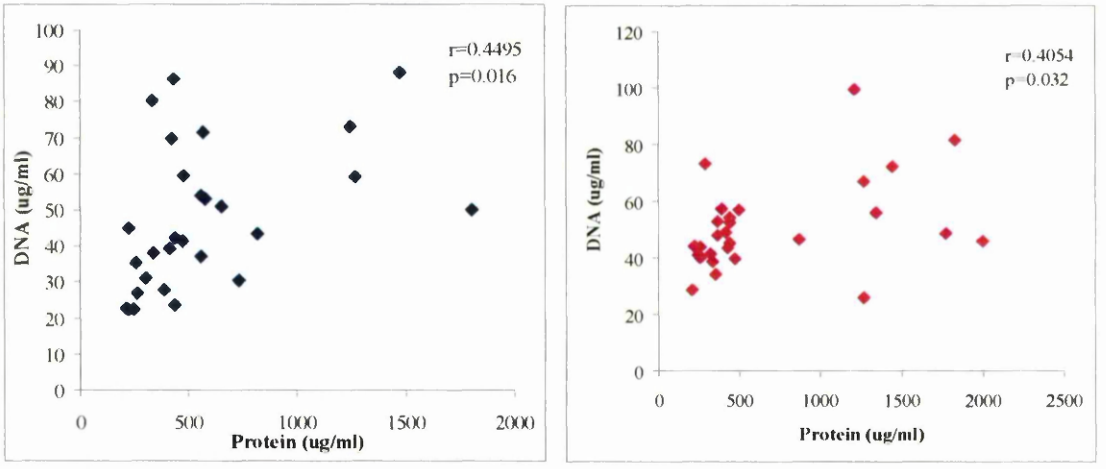


Figure 3.3.1.3c *Correlations between protein content and DNA content of cell lysates of fresh (blue) and cryopreserved (pink) trophoblast cells. The correlation coefficient (r) and significance (p) is as calculated using Pearson's bivariate pairwise correlation.*

3.3.2 Immunopurification of Trophoblasts

3.3.2.1 Screening Purified Cells Using Antibody Panel

Following isolation of trophoblasts from three different term placentae, cytospins were prepared using cells before and after immunopurification using HLA Class I coated magnetic beads (see section 2.2.2). Fluorescent immunocytochemistry was carried out on these cytospins using a panel of antibodies designed to detect and identify contaminating cells. Table 1.4.5.2 outlines the placental cell types each antibody reacts with. The reactivity of these antibodies was also tested on frozen term placental sections (figure 3.3.2.1a). It can be seen from this figure that the cytokeratin 7 antibody picks up only trophoblast cells, while the HLA class I, FSA and vimentin antibodies reacted with all placental cells except the trophoblast layer. The CD9 antibody showed very variable staining of stroma and blood vessel endothelium, with decidual staining being most evident. The CD163 antibody picked out specific stromal cells, which are likely to be hofbauer cells. The CD45 antibody failed to react with tissue sections probably because this is specific for blood leukocytes and there was very little blood on the frozen sections. The negative control sections showed no reactivity.

When used on cytospins, these antibodies showed that the contaminating cells were mainly blood leukocytes (CD45, HLA Class 1 and vimentin positive) with a minor

contribution from hofbauer cells (CD163, HLA Class 1 and vimentin positive) and other fibroblasts (HLA Class 1 and vimentin positive). Table 3.3.2.1b outlines the average result based on counting 4 fields of view on each cytospin from 3 pre and post purification cultures. These findings are also shown in figure 3.3.2.1b. Vimentin and cytokeratin antibodies were used to assess the purity of each subsequent cell isolation preparation.

Antibody	Culture 1		Culture 2		Culture 3	
	Pre	Post	Pre	Post	Pre	Post
Vimentin	7.25%	0.5%	7.5%	0	10.25%	0.5%
HLA Class I	8.25%	1%	6%	0	8.75%	0
CD9	0.5%	0	1.5%	0	1.25%	0.25%
CD45	8.25%	2%	5.75%	0	9.25%	0
CD163	0.25%	0	0	0	2.25%	0

Table 3.3.2.1b *Percentage positive staining of cytotrophoblast cytospins pre and post immunopurification using HLA Class I antibody coated beads.*

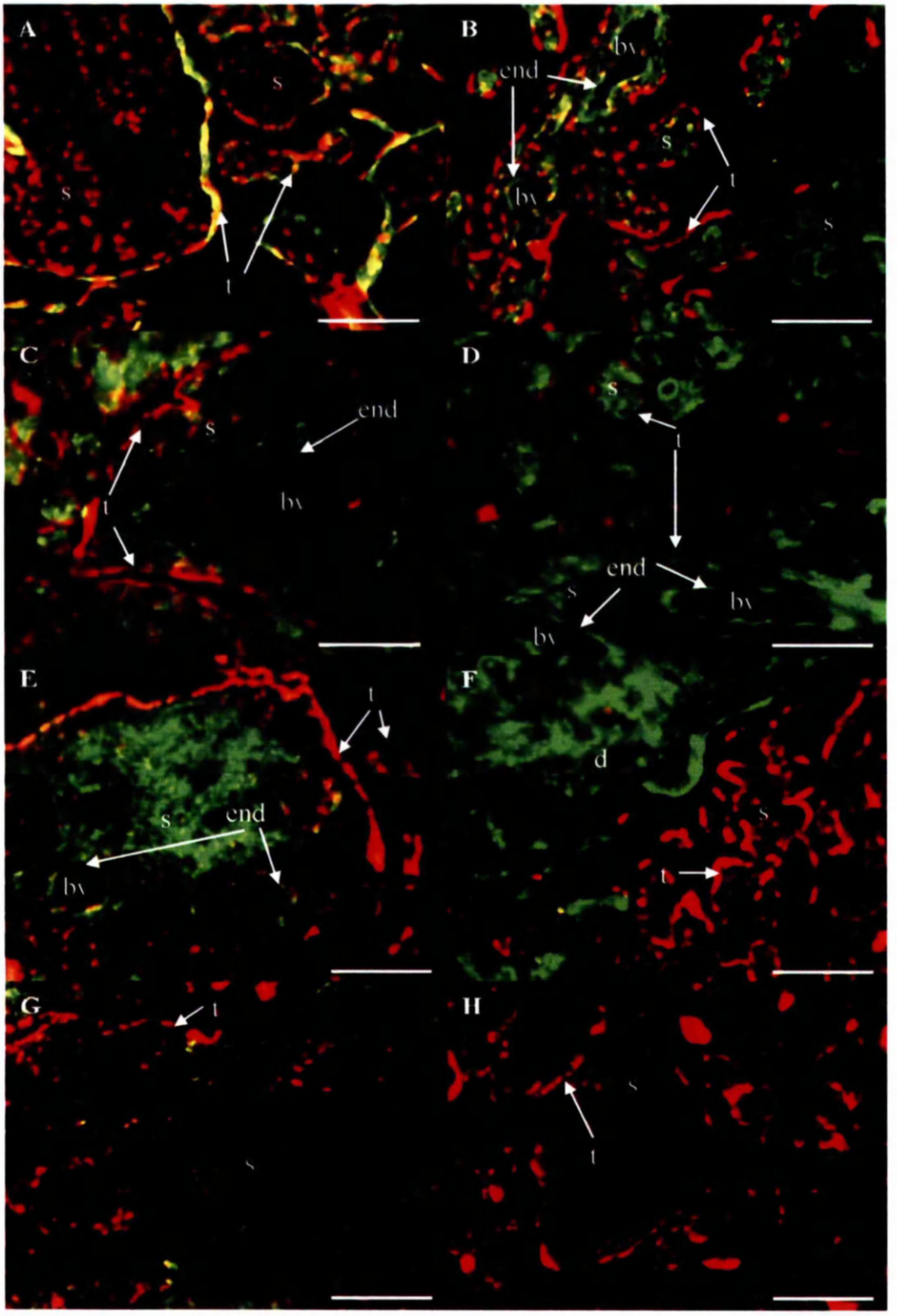


Figure 3.2.2.1a *Fluorescent IHC on frozen term placental sections with panel of antibodies used to assess trophoblast purity. Antibody staining is green and nuclei are counterstained red. Staining was carried out using antibodies against A-cytokeratin 7, B-vimentin, C-FSA, D-HLA Class I, E-CD9, F-CD9, G-CD163, H-negative control. Trophoblast layer (t), stroma (s), blood vessels (bv), blood vessel endothelium (end) and decidua (d) are labelled. Size bar represents 200μm.*

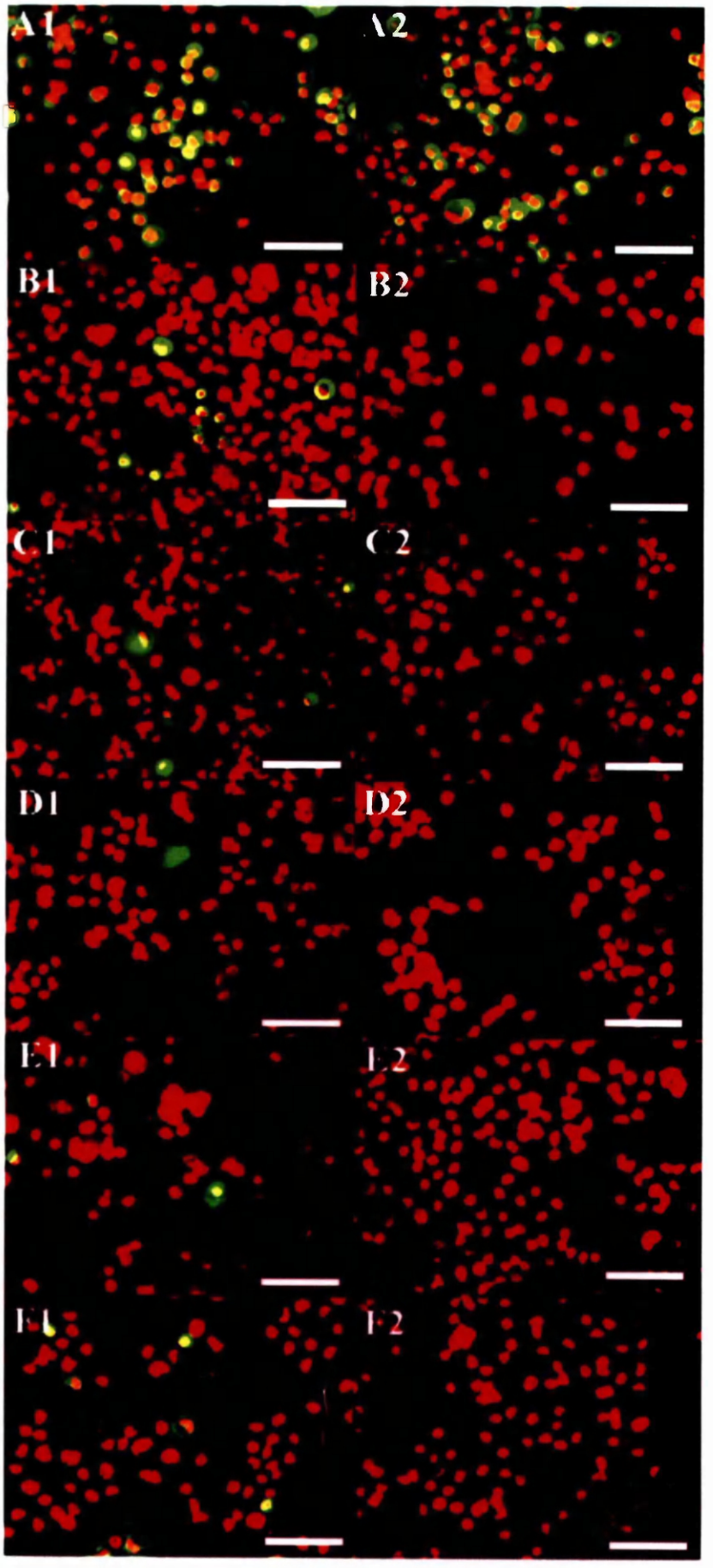


Figure 3.2.2.1b
Fluorescent ICC on pre (column1) and post (column2) purification cytospins of isolated placental trophoblasts. Antibody reactivity is green coloured and nuclei are counterstained red. Size bar represents 50μm.
A-cytokeratin 7,
B-vimentin,
C-HLA Class I,
D-CD163,
E-CD9,
F-CD45.

3.3.2.2 *Effects of Immunopurification on Syncytialisation*

The effects of immunopurification on hCG and PLAP production as well as morphological differentiation were studied. Figures 3.3.2.2a and 3.3.2.2b demonstrate that production of both proteins, and therefore syncytialisation, was not significantly altered by immunopurification (as tested by Wilcoxon Signed Ranks Test). This observation was also supported by no difference in morphological differentiation as observed in figures 3.3.2.2c and 3.3.2.2d which show the fusion of discrete cells into syncytial layers as marked by the disappearance of desmoplakin 1&2 staining between nuclei, and increased production of PLAP as syncytialisation progressed.

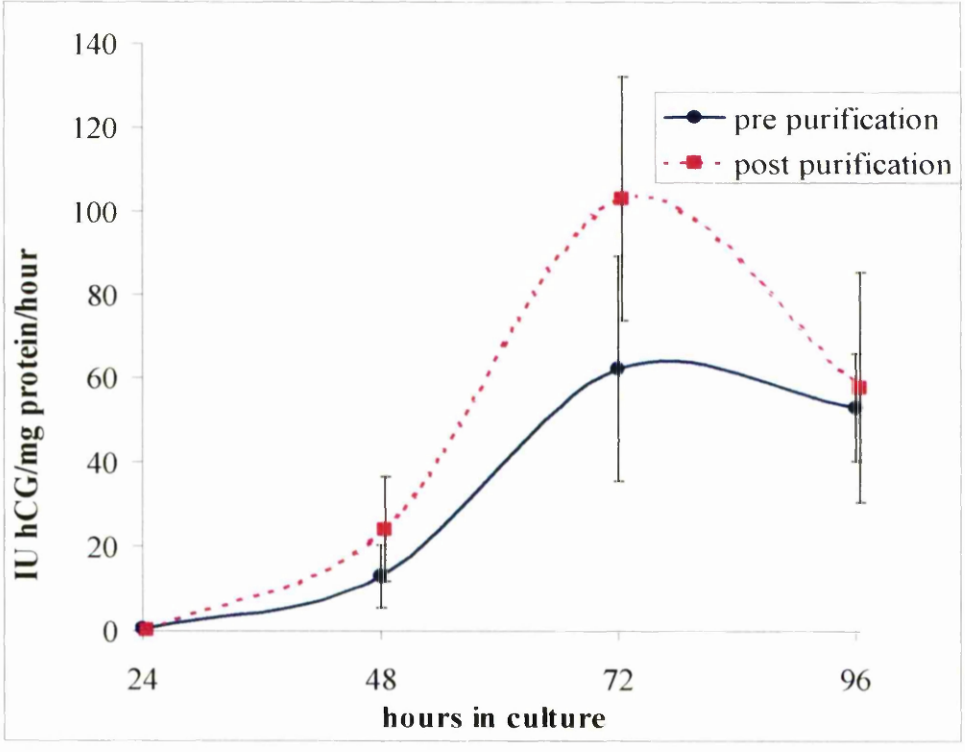


Figure 3.3.2.2a *Effects of immunopurification on the rate of hCG secretion. Results are expressed as a mean of 3 cultures ± SEM in IU hCG per mg protein per hour.*

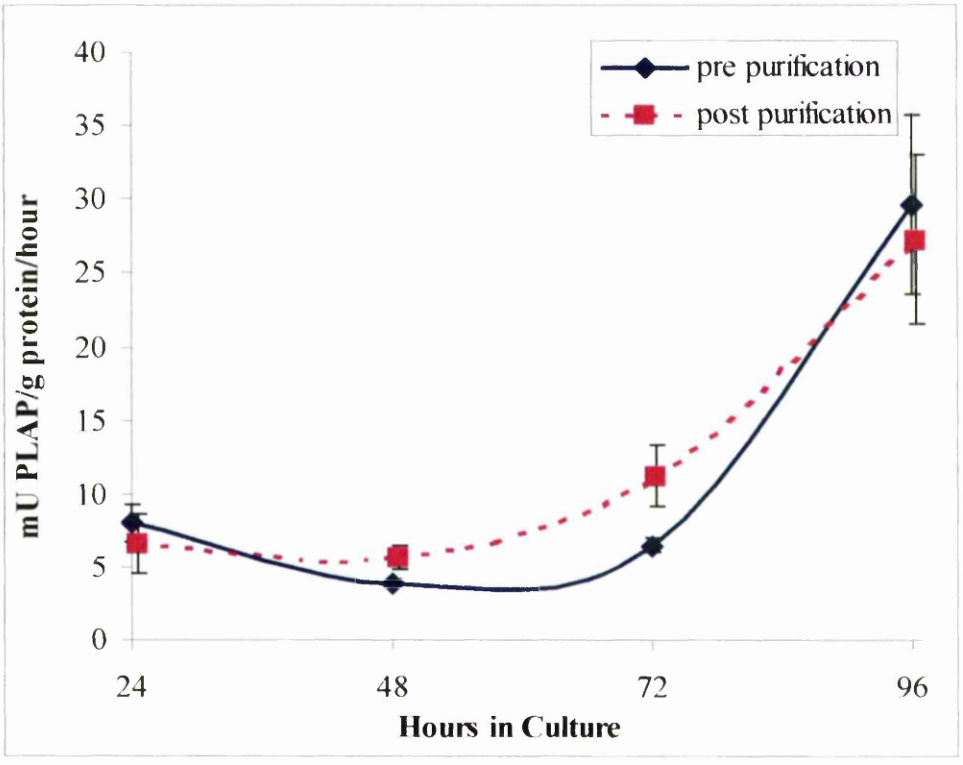


Figure 3.3.2.2b *Effects of immunopurification on the rate of PLAP secretion. Results are expressed as a mean of 3 cultures ± SEM in mU PLAP per mg protein per hour.*

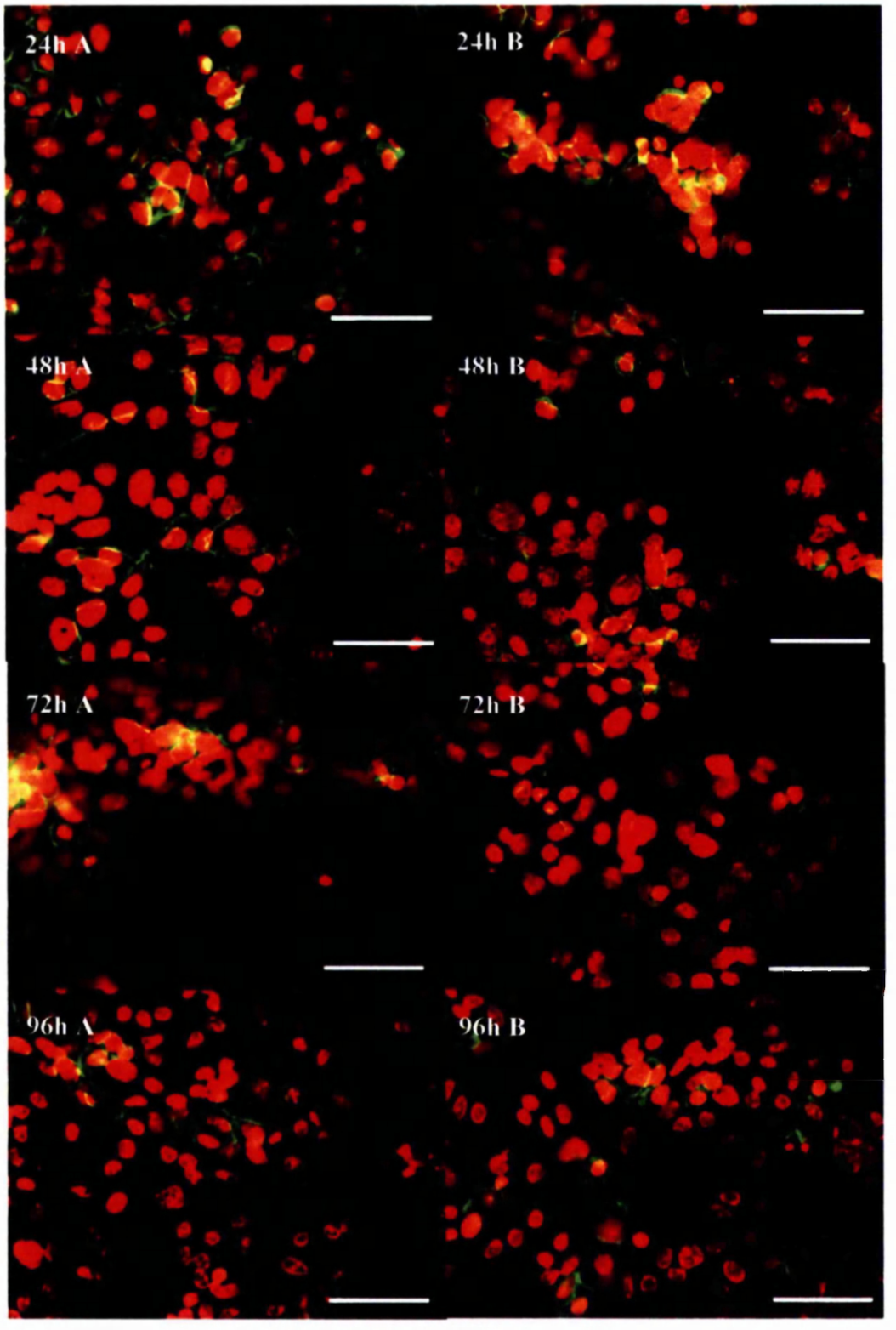


Figure 3.2.2.2c *Fluorescent ICC for desmoplakin 1&2 on pre (column A) and post (column B) purification placental trophoblasts, fixed at 24h intervals over a 96h culture period. Over the culture period staining for desmoplakin disappeared between some nuclei, which indicates formation of syncytium. Antibody reactivity is green coloured and nuclei are counterstained red. Size bar represents 50μm.*

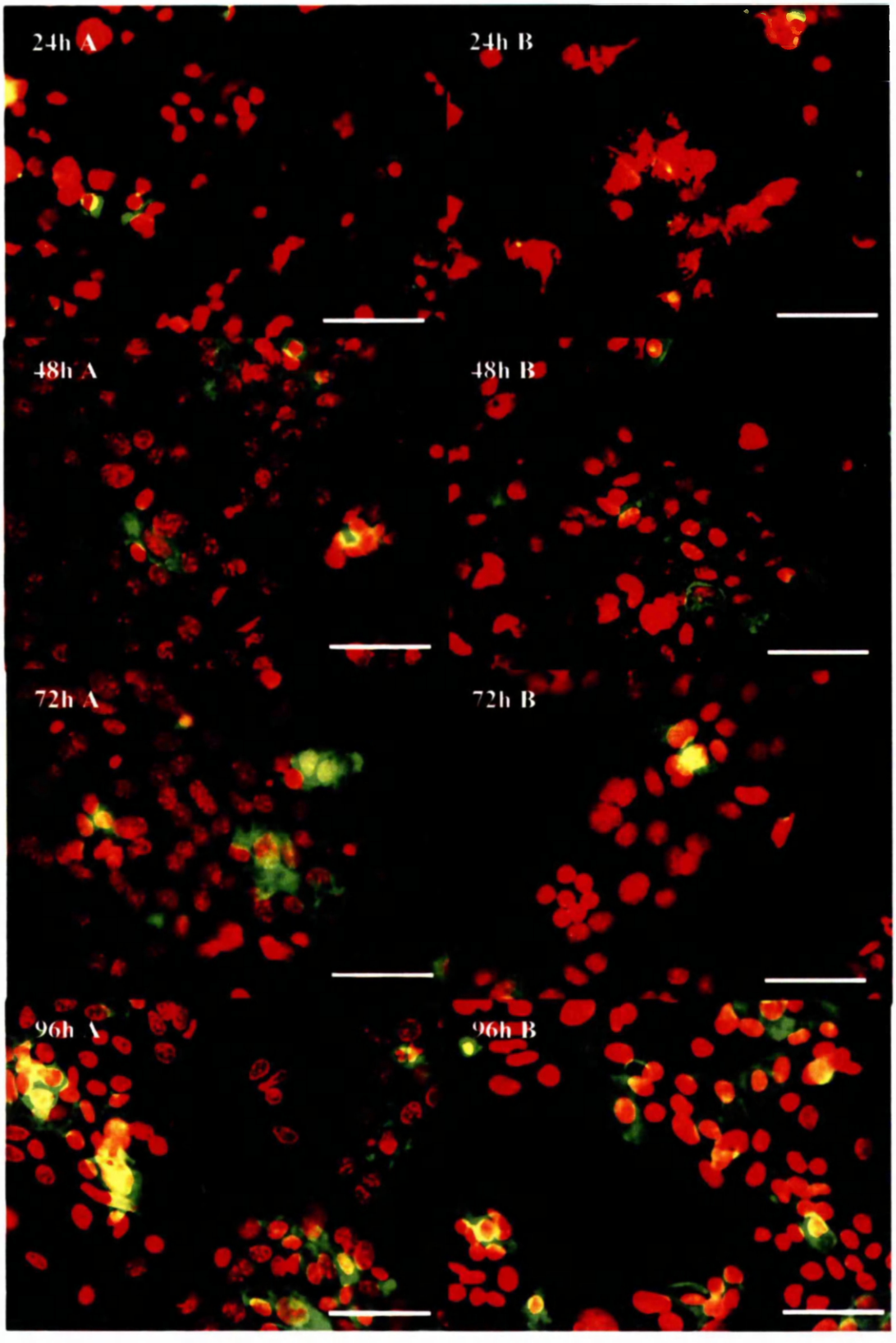


Figure 3.2.2.2d *Fluorescent ICC for PLAP on pre (column A) and post (column B) purification placental trophoblasts, fixed at 24h intervals over a 96h culture period. Over the culture period staining for PLAP increased, as syncytialisation proceeded. Antibody reactivity is green coloured and nuclei are counterstained red. Size bar represents 50μm.*

3.4 Challenging Trophoblasts with Interferon Alpha (IFNα)

3.4.1 Confirmation of Functional Interferon Receptor (IFN-R)

3.4.1.1 Localisation of IFN- R1 & R2 in Placental Sections

IFNα/β receptors (IFN-R1 & R2) were localised by immunohistochemistry on frozen sections from mid trimester control and DS placentae. Most placental cells expressed IFN-R in both term and mid trimester placentae (fig 3.4.1.1), although staining was most intense on trophoblast cells. Semi quantitative analysis of staining intensity revealed no significant difference in IFN-R staining intensity between control and DS sections.

3.4.1.2 Localisation of IFN- R1 & R2 in Isolated Trophoblasts

Fluorescent immunocytochemistry was carried out on acetone fixed trophoblast cytospins to confirm the presence of IFN-R1 & R2 on freshly isolated cells. Cells from four different placentae were investigated and all were found to express IFN-R on the cell surface (fig 3.4.1.2).

3.4.1.3 Confirmation of STAT1 Activation

The first event of IFNα/β receptor activation is phosphorylation of STAT 1. Protein isolated from cultured trophoblasts at 24h time points treated or untreated with IFNα, was analysed by western blotting to confirm the presence of activated (phosphorylated) STAT 1α and STAT 1β. A positive control cell lysate (Santa Cruz) was also run on western blotting gels. Bands of the expected sizes (84&91kDa) were observed in the positive control lane and the smaller (84kDa) band was present in the majority of cell lysates indicating the presence of activated STAT 1 (fig 3.4.1.3). Other non-specific bands of smaller proteins were observed in all lanes including the positive control.

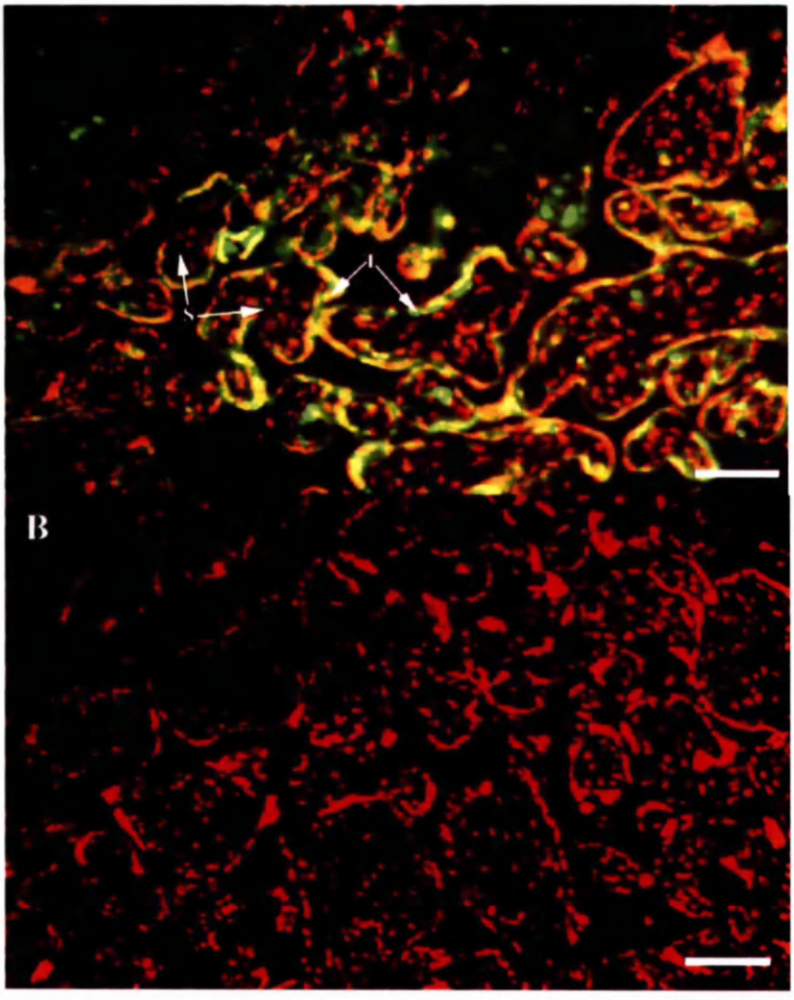


Figure 3.4.1.1
Fluorescent immunohistochemical localisation of IFN-R1 & R2 on term placental sections. Antibody reactivity is green coloured and nuclei are counterstained red. Trophoblast layer is indicated by T and stroma by S.
A= IFN-R1 & R2 localisation,
B=negative control,
Size bar =100μm.

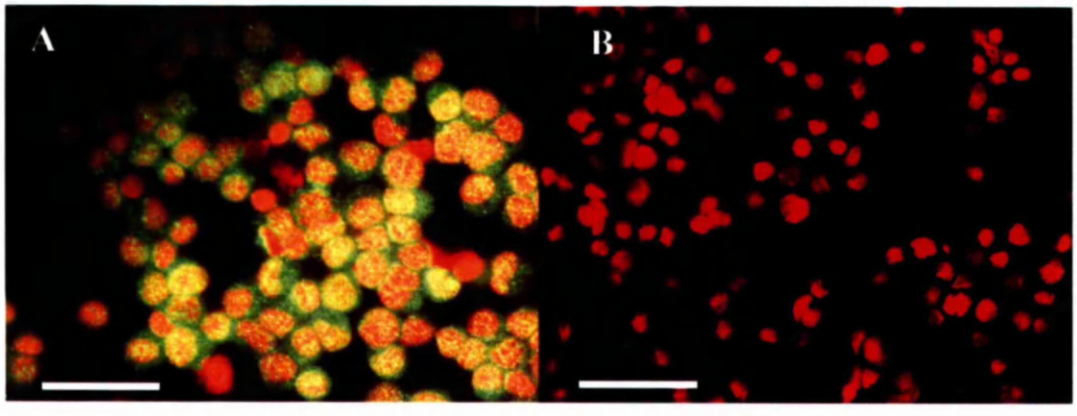


Figure 3.4.1.2 *Fluorescent ICC localisation of IFN-R1 & R2 on isolated trophoblast cells. Antibody reactivity is green coloured and nuclei are counterstained red.*
A=IFN-R1 & R2 localisation, B=negative control. Size bar =50μm.

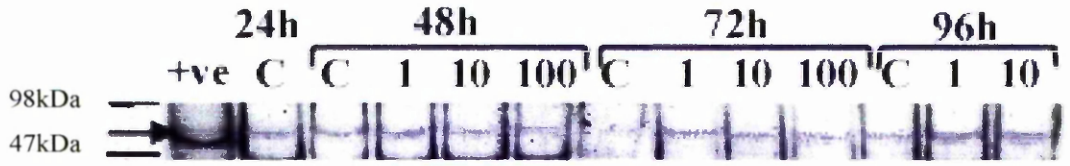


Figure 3.4.1.3 *Western blot for pSTAT 1 using cell lysates from trophoblast cultures treated or untreated with IFNα. Cell lysates were collected at 24h periods and had been cultured in the presence of IFNα at 1, 10 or 100U/ml. Negative control cultures (C) received no IFNα. Approximate band sizes in kDa are indicated as obtained from a size marker. A positive control (+ve) was also included.*

3.4.2 Effect of IFNα on hCG Production by Term Trophoblasts

3.4.2.1 hCG Secretion into Culture Medium

Trophoblasts isolated from six placentae were cultured in the presence and absence of various concentrations if IFNα. Culture medium was collected at 24h intervals and the quantity of hCG secreted (measured by immunoradiometric assay) was taken as a ratio of protein level of the cell extract from the appropriate wells. In all cases, the rate of hCG secretion increased to a peak at 72h after plating and declined until the end of the culture period. Addition of IFNα at different concentrations did not make a significant difference to the rate of hCG secretion at any time point (fig 3.4.2.1a). Addition of 100U/ml did, however, seem to cause the cells to peel from the culture surface after 96h in culture which meant it was difficult to accurately assess the quantity of hCG secreted under these conditions. The cumulative total quantity of hCG released into the medium over the 96h culture period did not significantly differ between the treatments (fig 3.4.2.1b).

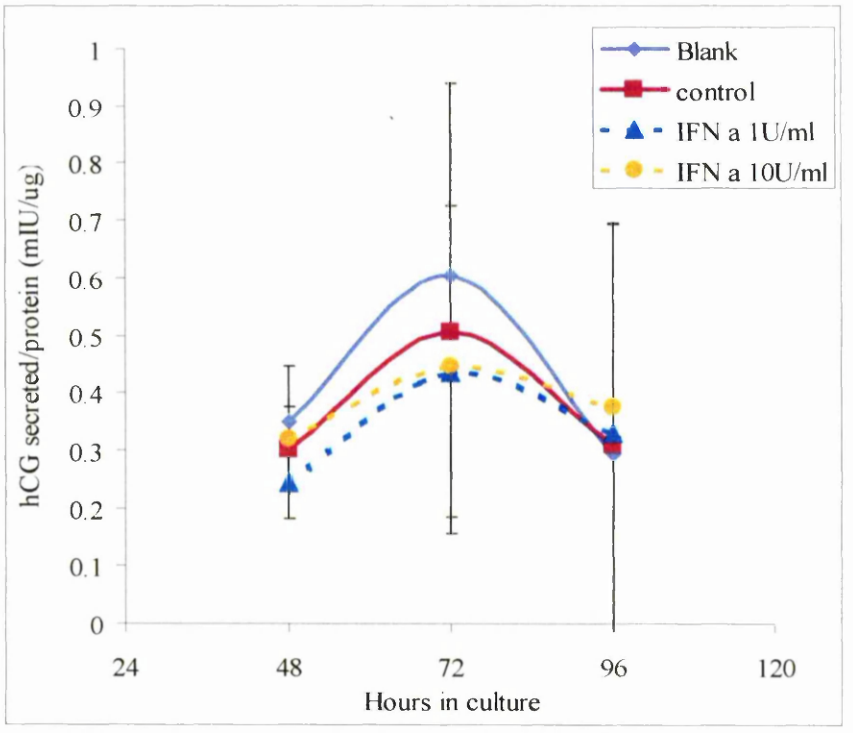


Figure 3.4.2.1a *Effects of IFNα treatment on the rate of hCG secretion. Results are expressed as a mean of 6 cultures in mIU hCG per μg protein per 24h period ± SEM. Blank=no addition, control=carrier added.*

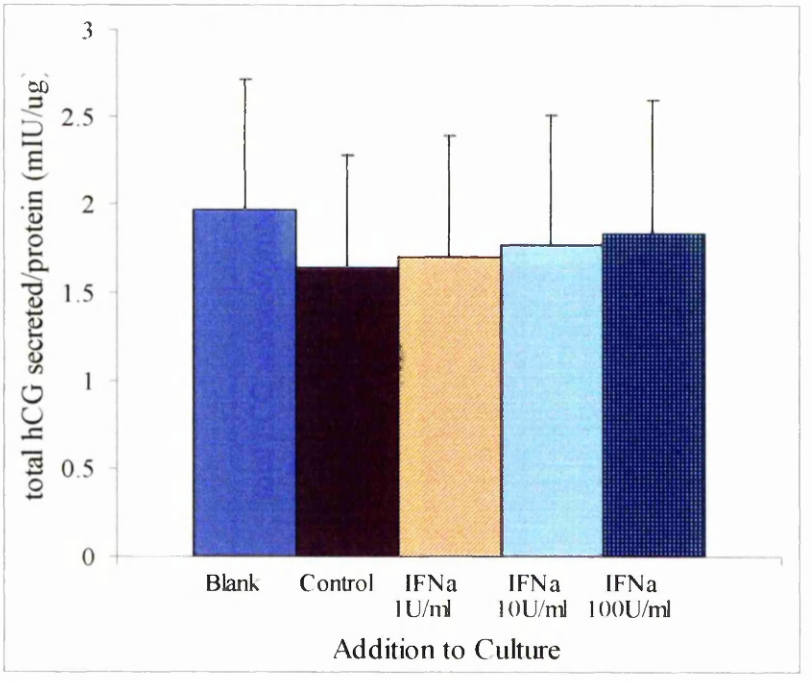


Figure 3.4.2.1b *Effects of IFNα treatment on the cumulative total amount of hCG secreted in the 96h culture period. Results are expressed as a mean of 6 cultures in mIU hCG per μg protein. Error bars indicate SEM. Blank=no addition, control=carrier added.*

3.4.2.2 *hCG in Trophoblast Cells*

From the same cells cultured in the presence and absence of IFNα, total protein was extracted from the scraped cells. The hCG concentration was determined in these protein extract using an immunoradiometric assay and was expressed as a ratio of the total protein concentration. As with the rate of hCG secretion, no significant difference in rate of hCG production or the cumulative total amount of hCG present in the cells over the culture period was observed (fig 3.4.2.2a, fig 3.4.2.2b).

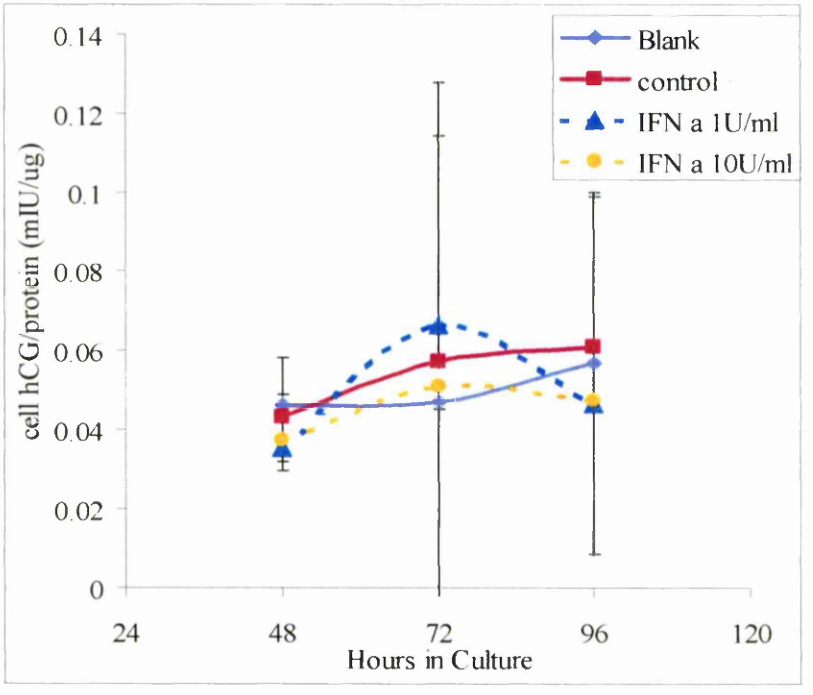


Figure 3.4.2.2a *Effects of IFNα treatment on the quantity of hCG in trophoblast cells. Results are expressed as a mean of 6 cultures in mIU hCG per μg protein per 24h period ± SEM. Blank=no addition, control=carrier added.*

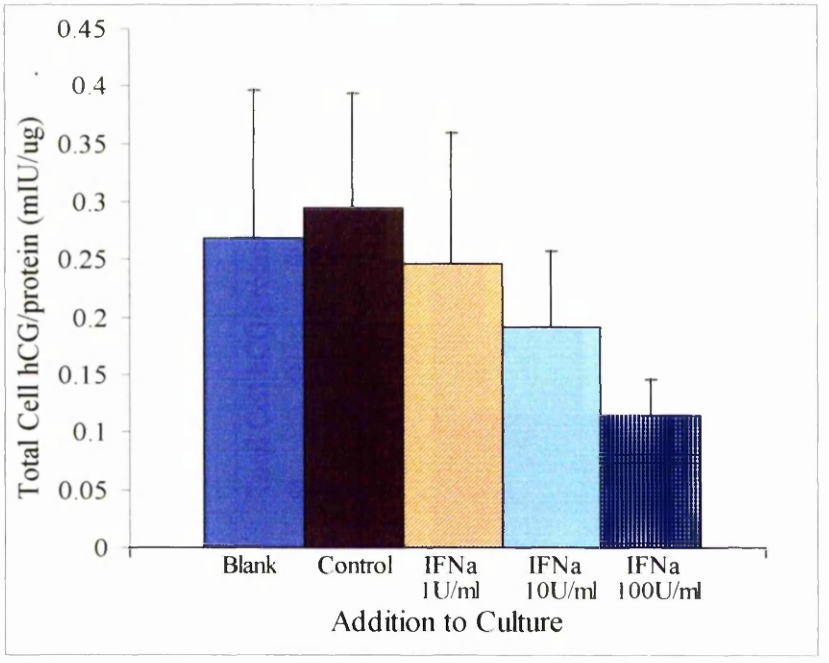


Figure 3.4.2.2b *Effects of IFNα treatment on the cumulative total amount of hCG in trophoblast cells over the 96h culture period. Results are expressed as a mean of 6 cultures in mIU hCG per μg protein. Blank=no addition, control=carrier added.*

3.3.2.3 hCG mRNA Levels

From the same cells cultured in the presence and absence of IFNα, mRNA was extracted from the scraped cells. The quantity of hCG mRNA and PPIA mRNA in each sample was assessed using real time quantitative PCR. The rate of hCG mRNA production, corrected against the housekeeping gene PPIA and expressed relative to a calibrator sample, was determined for each treatment (expressed as $2^{-\Delta\Delta Ct}$). There was no significant difference in the rate of hCG mRNA production when the cells were treated with IFNα (fig 3.4.2.3).

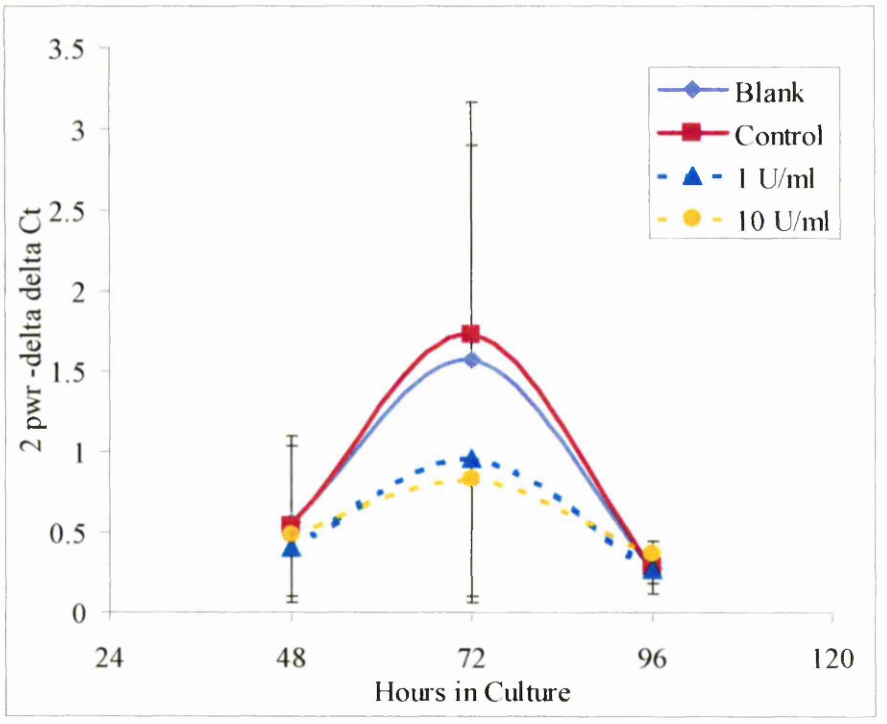


Figure 3.4.2.3 *Effects of IFNα treatment on the rate of hCG mRNA production. Results are expressed are the mean of 6 cultures as* $2^{-\Delta\Delta Ct}$ *which expresses hCG mRNA levels relative to PPIA levels per 24h period ± SEM. Blank=no addition, control=carrier added.*

Section 4

Discussion

4.1 Inhibin and Activin

4.1.1. Inhibin-A

This study has demonstrated that both the maternal serum and placental levels of inhibin-A are elevated in DS pregnancies. These results are in agreement with previous studies on maternal serum which reported an elevation in the median DS inhibin-A level to between 1.6 and 3.65 MoM (Spencer et al. 1993; Cuckle et al. 1994; Cuckle et al. 1995; Aitken et al. 1996; Wald et al. 1996; Wallace et al. 1996; Wallace et al. 1996; Lambert-Messerlian et al. 1996a; Lambert-Messerlian et al. 1998a; Debiève et al. 2001). The results of the earlier studies (Spencer et al. 1993; Cuckle et al. 1994; Cuckle et al. 1995) were generated using an older inhibin-A assay that detected free subunits of inhibin-A as well as the intact dimer. It is therefore more appropriate to compare the current results with those using the dimeric inhibin-A assay (Groome, 1991; Groome and O'Brien, 1993) as used in the present study. The DS median MoM of 2.06 reported in the current study is towards the higher end of the range of those previously reported. The reason for this observation may be that most of the serum samples used in this study had elevated hCG (Newby et al. 1997) because they were identified by prenatal screening. Serum hCG and inhibin-A show a degree of correlation (Aitken et al. 1996) therefore, a serum sample with high hCG levels would also be expected to have an elevated inhibin-A level.

The significant elevation in DS placental inhibin-A levels to 1.46MoM in the current study contrasts with the results recently reported by Debiève *et al* (2001). In the latter study, which was based on placental protein extracts from only 6 DS placentae collected between 15 and 32 weeks of gestation, no significant difference was found in inhibin-A or pro αC levels in DS placentae. There was however, a trend towards elevated levels of both inhibin-A (1.29MoM) and pro αC (2.66MoM). The reason why these differences were found not to be statistically significant could be due to the small number of samples in the study group, or the wide range of gestations with some samples obtained in the third trimester when placental inhibin-A levels may not show markedly elevated levels in DS pregnancies.

There was no significant correlation between placental and maternal serum levels of inhibin-A, contrasting with the association previously reported between placental and serum levels of hCG (Newby et al. 1997). To investigate whether the difference in

gestation between the collection of a serum screening sample and time of TOP could be the reason for this lack of correlation, a series of placental homogenates and matched serum samples taken just prior to TOP were investigated. As can be seen from table 3.1.6.1a, this produced a significant correlation between maternal serum and placental levels of inhibin-A, as would be expected when a placental product is secreted directly into the maternal serum.

Inhibin-A is already used as a DS screening marker in some areas in combination with AFP, hCG and in some cases UE_3. It has been reported that the addition of inhibin-A to these combined marker tests gives an improvement in the detection rate of DS pregnancies by around 15-20% when compared with the use of maternal age, AFP and either ihCG or fβ-hCG with or without UE_3. The detection rate for AFP, ihCG or fβ-hCG and age screening with a 5% false positive is around 54% (Aitken et al. 1996; Wald et al. 1996). The addition of inhibin-A increases this detection rate to between 67% (Wald et al. 1996) and 75% (Aitken et al. 1996) for a 5% false positive. Although a slight increase in the first trimester maternal serum level of inhibin-A has been reported in DS pregnancies (Wallace et al. 1994; Aitken et al. 1996) the detection rate is not high enough to make this a useful screening marker in the first trimester unlike PAPP-A or fβ-hCG.

Amniotic fluid levels of inhibin-A have been previously reported as being reduced in DS pregnancies (Wallace et al. 1997) which is the opposite of observations in maternal serum and placental extracts. This may suggest that the placenta is not the major source of amniotic fluid inhibin-A; alternatively there could be altered placental transport of inhibin-A from the placenta in DS pregnancies.

Alterations in circulating levels of inhibin-A have also been reported in other complications of pregnancy including pre-eclampsia. It has been noted that in the third trimester of pregnancy, women with preeclampsia have significantly elevated maternal serum concentrations of inhibin-A and in the second trimester significantly increased maternal serum inhibin-A is predictive of preeclampsia in later pregnancy (Muttukrishna et al. 1997; Cuckle et al. 1998). This has been suggested as being indicative of trophoblast hypoplasia. In contrast to the elevations in maternal serum inhibin-A levels in DS and preeclamptic pregnancies, trisomy 18 and trisomy 13

affected pregnancies show reductions in maternal serum levels of inhibin-A (Crossley et al. 1991; Lambert-Messerlian et al. 1998b).

4.1.2 Activin-A

As with inhibin-A, a clear elevation of both placental (1.62MoM) and maternal serum (1.26MoM) activin-A is evident in DS pregnancies. These results agree with the study that reported a significant increase in the DS maternal serum level of activin-A to 1.25 MoM ($p<0.01$) (Lambert-Messerlian et al. 1996b). These results contrast with the small reduction in maternal serum activin-A levels reported in a second study by the same group (Lambert-Messerlian et al. 1998a). A study by Cuckle *et al* (1999a) also found maternal serum levels of activin-A to be elevated to 1.19 MoM in DS pregnancies, but concluded that the distribution of the control and DS levels would not make this a suitable screening marker for DS pregnancies. Significantly elevated maternal serum activin-A levels have also been reported in the first trimester of DS pregnancies to 1.36MoM (Spencer et al. 2001). Again, the overlap in the distribution of control and DS levels make this an unsuitable serum screening marker.

Debiève *et al* (2001) noted a non-significant increase in placental activin-A levels in DS placentae to 1.45MoM. As noted above sample group size or gestational range of the samples could account for the lack of statistical difference between sample groups. Increased maternal serum activin-A levels have also been observed in other disorders of pregnancy, including pre-eclampsia, gestational diabetes and pre-term labour (Petraglia et al. 1995a; Petraglia et al. 1995b; Petraglia et al. 1999). Although these conditions are not associated with DS pregnancy it suggests that alterations in the normal function of the placenta and of the fetus are associated with altered maternal serum levels of this marker. As with the inhibin-A data, no significant correlation was observed between the serum and placental levels of activin-A. Again, pre-termination serum samples were compared with placental levels, however, unlike inhibin-A, this did not produce a significant correlation (table 3.1.6.1a).

As noted earlier, activin-A is unlikely to prove a useful screening marker of DS in either the first or the second trimester of pregnancy, despite a significant elevation in the level of this marker in the maternal serum of DS pregnancies having been reported in the

current and other studies (Cuckle et al. 1999a; Spencer et al. 2001). This is because the large overlap in the distribution of control and DS maternal serum levels of activin-A in both trimesters mean that to give a false positive rate of 5% a low detection rate would be achieved. In the second trimester of pregnancy, there was a significant correlation between the maternal serum levels of inhibin-A and activin-A shown in the current study and by Cuckle *et al* (1999a). This means that, even if there was less of an overlap between control and DS cases, activin-A would be unlikely to add to a screening study already including inhibin-A. In the first trimester, a significant association between activin-A and both PAPP-A and fβ-hCG was found (Spencer et al. 2001) again indicating that this marker would be unlikely to increase the detection rate of the current screening strategy.

4.1.3 Immunohistochemical Analysis of α and β_A Subunits

4.1.3.1 Immunolocalisation of Subunits

Inhibin and activin α and β_A subunits were localised by immunohistochemistry (fig 3.1.1.1a & 3.1.1.1b). The observed staining pattern for both the α and β_A subunits agrees with the immunohistochemical localisation of the activin-A dimer reported by Rabinovici *et al* (Rabinovici et al. 1992) who also noted positive staining in both cytotrophoblast cells and syncytiotrophoblast, as well as some stromal cells. Two studies (Petraglia et al. 1991; Petraglia et al. 1992) detected α and β_A subunits in all trophoblastic cells a finding that the present study has confirmed. McCluggage *et al* (1998) studied the localisation of the α subunits of inhibin using antibodies from the same source as those used in the present study, and found this subunit to be localised to syncytiotrophoblasts, but not cytotrophoblast cells. A similar result was reported by Minami *et al* (1992), who noted positive staining for both α and β_A subunits in the syncytial layer, but not in cytotrophoblast cells. The reason for the variations in subunit localisation reported by the different studies is unclear. There appears to be no association between the gestational range of samples or the source of specific antibody with the reported subunit localisation.

A study (Debieve and Thomas, 2000) on isolated trophoblast cells examined the stage in culture when mRNA for the α and β_A subunits was detectable. It was noted that freshly isolated cytotrophoblast cells expressed α subunit mRNA but showed very little β_A subunit expression. The expression of α subunits rose steadily until a peak at 72h in

culture then began to decline. In contrast β_A subunit expression rose quickly to a peak at 48h in culture with the levels remaining constant until 120h when levels declined. It was suggested that inhibin-A production could therefore be considered a marker of syncytialisation, while activin-A seems to be produced during cell aggregation prior to syncytialisation (Debieve and Thomas, 2000). These results provide support for the reports that suggested inhibin α and β_A subunits could be immunolocalised to both cytotrophoblast cells and syncytiotrophoblast cells, since mRNA for both subunits was detectable after 24h in culture at which point most cells are still mononucleated.

From these studies on the localisation of inhibin-A and activin-A in the placenta, it is probably fair to conclude that the subunits of inhibin-A and activin-A are produced by both cytotrophoblast cells and the syncytiotrophoblast. These cells are the primary site of production of most placental products used as maternal serum markers of DS e.g. hCG (Hay, 1988; Maruo et al. 1992) and PAPP-A (Tornehave et al. 1984). This observation indicates that when studying placental expression of DS markers, cytotrophoblast cells are probably the most useful to isolate and study in culture as they fuse to form syncytium.

4.1.3.2 Comparison of Staining Intensity

The semi-quantitative analysis of staining intensity (table 3.1.3.2) suggests that the increased levels of dimeric inhibin-A and activin-A in DS pregnancies are due to excess production of both α and β_A subunits. Lambert-Messerlian *et al* (1998a) found that α subunit but not β_A subunit mRNA production was elevated in DS placental extracts. Using a PCR based method of measuring subunit production as Lambert-Messerlian *et al* (1998a) did is more selective than quantitative immunohistochemistry which detects not only the free subunits but also the intact dimers. Using end point RT-PCR can however prove difficult to optimise so that mRNA levels in samples are measured during the logarithmic stage of the PCR reaction. The study of Debiève *et al* (2001), found no difference in either α or β_A subunit mRNA levels in placental tissues from DS pregnancies again using end point RT-PCR analysis. This agrees with the β_A subunit results of Lambert-Messerlian *et al* (1998) but differs from the results obtained for the α subunit mRNA levels reported in that study. A possible reason why slightly different results have been reported could be due to the difference in gestational range of the samples used in each study. The Debiève study included some third trimester samples

while the Lambert-Messerlian study included samples collected only from the second trimester.

A similar study on the IHC staining intensity of inhibin α and β_A subunits in placental tissues has been carried out on preeclamptic and control tissues (Jackson et al. 2000). The staining for both subunits was significantly greater in the preeclamptic samples when compared with controls. This suggests that the increased maternal serum levels of both inhibin-A and activin-A observed in preeclamptic patients are due to increased placental production of both factors (Jackson et al. 2000), similar to the conclusion drawn in the current study about increased maternal serum levels of inhibin-A and activin-A being due to increased placental production in DS.

As can be seen from table 3.1.3.2, the DS samples from earlier gestations were more likely to show a positive difference than those from the later gestations, particularly in the case of alpha subunit staining. This observation matches the trend towards higher placental inhibin-A and activin-A MoMs at the earlier gestations studied (fig 3.1.1.1b & 3.1.2.1b). This may indicate that the extent of the elevation of inhibin-A and activin-A levels in DS placental tissues is gestation dependent. Evidence of this temporal variation can be found for maternal serum levels of inhibin-A in the study by Aitken *et al* (1996). Similar gestation dependent variations have been reported for both intact and free beta hCG in maternal serum from DS pregnancies (Aitken et al. 1993; Berry et al. 1997). PAPP-A levels have also been shown to differ through gestation with first trimester DS levels lower than normal while second trimester DS levels no different from those in the normal situation (Berry et al. 1997).

An obvious drawback when using IHC staining intensity as a measure of protein levels in tissues is the inherent inaccuracy of using a subjective rather than a strictly quantitative method of ranking the degree of staining. While immunoassay or RT-PCR methods provide an absolute result, the manual assessment of staining intensity is subjective and can vary depending on the person scoring and if all areas of a section have been viewed. In the current study, differences between and within scorer were analysed to counter possible inaccuracies. Even with these controls in place, the results of IHC staining intensity experiments should be treated as an indication of trends in differences in protein expression rather than precise results like those obtained from ELISA or RT-PCR experiments. The main advantage of IHC is of

course the accuracy of tissue specific localisation of the protein in tissues, information that cannot be provided by strictly quantitative methods.

4.1.4 Relationship Between Inhibin-A, Activin-A and hCG

To investigate the relationship between inhibin-A, activin-A and hCG levels, correlations between levels of these analytes were investigated in placental and serum samples. Both free beta hCG and intact hCG levels had previously been measured and reported in a selection of these samples by Newby *et al* (1997). As can be seen in table 3.1.6.1b, a significant correlation between control placental inhibin-A and fβ-hCG was observed, with a similar, but not statistically significant trend between inhibin-A and ihCG. These correlations are similar to those previously reported in maternal serum for the same markers (Aitken et al. 1996). In DS placental homogenates, no such correlation was observed between inhibin-A and fβ-hCG, although ihCG showed a similar trend to that observed in control placentae. In the case of DS serum samples, correlation coefficients between inhibin-A and either ihCG or fβ-hCG were similar to those reported by Aitken *et al* (1996), however they did not reach statistical significance like those reported previously. This could be due to the much smaller sample group used in the current study.

Interestingly, activin-A levels were found only to correlate with ihCG and fβ-hCG in DS serum samples, with no correlation evident in the placenta. Cuckle *et al* (1999) examined correlations between activin-A and fβ-hCG levels in maternal serum, and found no correlation in DS samples, but observed a significant correlation in control samples. Although no definitive reason can be suggested why correlation has been observed in serum samples but not placental samples, it is postulated that different follistatin binding of activin-A in the maternal and placental compartments could perhaps contribute to this effect. It is postulated that the majority of the increased levels of activin-A in the placenta is bound to follistatin, and is therefore biologically inactive. Only unbound activin-A would be free to cross into the maternal circulation, where the circulating form of follistatin would then bind it. This would explain the lack of correlation between placental and maternal serum levels of total activin-A. If this situation is compared with that of inhibin-A, the highly significant correlation between placental and pre-termination maternal serum levels of this analyte suggests that unlike activin-A, it is secreted directly into the maternal serum. Like the inhibin-A data, a

significant correlation between placental and serum levels of hCG was reported previously (Newby et al. 1997). The hypothesis that activin-A is held in the placenta, while inhibin-A is secreted upon production may also account for the smaller increase in maternal serum activin-A levels when compared with the elevation in serum inhibin-A levels (1.26 MoM compared to 2.06 MoM) found in the present study. Another observation that could support this theory is the greater increase in placental levels of activin-A when compared with inhibin-A (1.62 MoM compared to 1.46 MoM). This hypothesis may also explain the lack of correlation between activin-A and hCG levels in the placenta. If only the free form of activin-A may stimulate hCG production, then only free activin-A levels, and not total activin-A levels would be expected to correlate with fβ-hCG and ihCG levels. Since hCG acts to stimulate inhibin-A production, a degree of correlation would be expected between either ihCG or fβ-hCG and inhibin-A levels. In this study, a significant correlation between fβ-hCG and inhibin-A was observed only in control placentae. However, ihCG and inhibin-A showed a degree of correlation similar to that of activin-A and inhibin-A in the placenta, but in this case it was not statistically significant.

In maternal serum, inhibin-A, activin-A and both forms of hCG all showed a degree of correlation with each other, although this did not always reach statistical significance. It is speculated that this is due to an evenly upregulated secretion of inhibin-A, activin-A, and both forms of hCG from the placenta of DS pregnancies, rather than any of these analytes possessing a particular endocrine function. The small number of samples used to calculate these correlation coefficients, do however, make it difficult to draw any definitive conclusions. Further work, perhaps studying the distribution and levels of follistatin subtypes in the placenta and maternal serum, would be required to elucidate the mechanisms of activin-A storage and transport. One group (Cuckle et al. 1999a) analysed follistatin concentrations in the maternal serum of DS and control pregnancies in the second trimester. They found no significant difference in the level of follistatin between groups. This group used an assay that has a high affinity for follistatin 288 (membrane bound form) but has considerable cross reactivity with follistatin 315 (circulating form). It is likely that since follistatin 315 is the major form in serum that it will have provided a substantial contribution to the measured concentration of follistatin. To carry out more detailed studies, specific assays for both follistatin subtypes would be required. Future studies perhaps examining the level and distribution of follistatin 288 in control and DS placentae may provide further insights into the

mechanisms behind elevated maternal serum concentrations of inhibin-A and activin-A in DS pregnancies.

4.1.5 Conclusions: Inhibin and Activin Study

This study provides evidence that the elevation in both activin-A and inhibin-A in maternal serum from DS pregnancies may be due to overproduction of the proteins rather than increased transport from the fetal to maternal compartments. The observed correlation between activin-A and inhibin-A levels in placental extracts and a similar trend in serum samples could suggest one of two reasons for the increased hCG, inhibin-A and activin-A production observed in DS pregnancies. It could be that an independent factor (possibly coded for by a gene on chromosome 21) is increasing the production of activin-A, which would in turn lead to an increase in hCG production. The increased hCG levels would then be expected to up-regulate the rate of inhibin-A production as part of a negative feed back loop (fig 4.1.5).

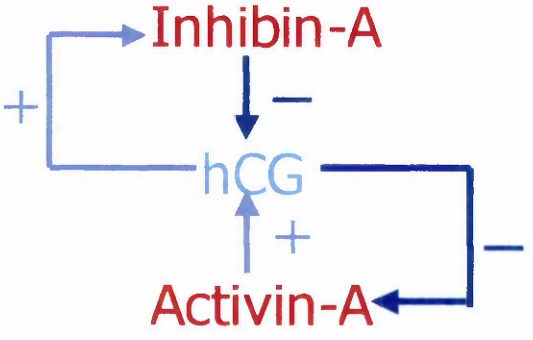


Figure 4.1.5 *Diagram of the negative feedback loop involving inhibin-A, activin-A and hCG. + indicates stimulation of production by one of the factors and - indicates inhibition of production*

Alternatively, the production of the β_A subunit of inhibin-A and activin-A could be upregulated by a different mechanism which would lead to increased production of both glycoproteins. Lambert-Messerlian *et al* (1998a) postulated that the observed increase in inhibin-A levels was due to upregulation in α-subunit production, while increased β_A subunit expression did not contribute to altered inhibin-A levels in the maternal serum of DS pregnancies. This was supported by their observation that neither β_A subunit mRNA nor activin-A production was altered. However, more β_A subunit would be required to maintain activin-A production since inhibin-A, and therefore the requirement for β_A subunits, is increased in DS pregnancies.

It is clear from the present and other studies that, despite the significant elevation in inhibin-A and activin-A in the maternal serum of DS pregnancies, inhibin-A but not activin-A is a useful marker of DS pregnancies in the second trimester. Increased placental production of these proteins, probably by trophoblast cells, is the most likely reason behind the observed elevation in maternal serum inhibin-A and activin-A. The correlation between inhibin-A/activin-A and hCG in the placenta suggests that the elevation in the production of hCG by the DS placenta is related to elevated inhibin-A and/or activin-A production. This relationship could be due to a negative feedback mechanism or an independent factor that increase the production of inhibin-A, activin-A and hCG.

4.2 Growth Factors

4.2.1 Epidermal Growth Factor

This study is the first to show that maternal urine and amniotic fluid levels of EGF are significantly lower than normal in DS pregnancies, while maternal serum levels are unaltered. Significantly reduced urinary but unaltered serum EGF levels was a surprising observation, since urine is a filtrate of the circulation. In this study significantly lower levels of EGF were also noted in amniotic fluid. Other markers show different trends in variation between amniotic fluid and maternal serum. For example, PLAP levels are reduced in amniotic fluid from DS pregnancies (Newby et al. 1997), but are normal in maternal serum, and the inhibin-A concentration is reduced in amniotic fluid but elevated in maternal serum of DS pregnancies (Wallace et al. 1997). This may indicate different sources of EGF in maternal urine, maternal serum and amniotic fluid. Alternatively, the rapid clearance of EGF from the maternal circulation may be such that it makes differences in levels impossible to detect in serum. Maternal serum samples used in this study were all from the second trimester of pregnancy, while the urine samples were obtained from first and second trimesters. Our data suggest that DS urinary EGF MoM values may be lower in the first trimester of pregnancy than in the second (fig 3.1.4.3b), which may indicate that this is a transient alteration of levels, mainly observed in the first trimester of pregnancy. Low maternal urine and amniotic fluid EGF levels have previously been noted in pregnancies affected by intrauterine growth retardation (IUGR) (Hofmann et al. 1988; Lindqvist et al. 1999). A review of post-mortem reports for the DS fetuses investigated in the present study found no evidence of IUGR, consistent with previous observations (MacAfee et al. 1970; Kučera, 1971; Wald et al. 1993). One study has failed to find placental expression of EGF (Jokhi et al. 1997), but both EGF and its receptors have been localised to trophoblastic cells of the placenta (Ladines-Llave et al. 1991). This suggests that EGF may have an endocrine role in the development of the placenta.

Reasons why EGF levels should be reduced in the amniotic fluid of DS pregnancies are speculative since the precise origin of this growth factor is unclear. It is known that EGF is present in fetal skin (Poulsen et al. 1996), and also that the dermis of fetal skin is abnormally thickened due to increased collagen type VI production in DS pregnancies (von Kaisenberg et al. 1998). Such abnormal skin development may be

associated with altered secretion of EGF, leading to changes in amniotic fluid levels of this growth factor. Alternatively, reduced excretion of EGF in urine by DS fetuses would lead to altered amniotic fluid levels of EGF since fetal urine is the principal contributor to the AF pool around the end of the first trimester.

4.2.2 Transforming Growth Factor Beta 1

This is the first study to show that placental and amniotic fluid levels of $TGF\beta_1$ are significantly lower than normal in DS pregnancies, while maternal serum levels are unaltered. As with EGF, it is unclear why amniotic fluid and placental levels of $TGF\beta_1$ are reduced in DS pregnancies. These results contrast with a report that total bioactive TGFβ levels are elevated in the amniotic fluid of DS pregnancies (Bromage et al. 2000). The latter study was carried out using a bioassay, which was based on the ability of TGFβ to inhibit the proliferation of mink lung fibroblasts. The current study measured total $TGF\beta_1$ concentration, that is bioactive and latent forms and found a significant reduction in placental and amniotic fluid levels. The study by Bromage *et al* (2000) found a significant increase in bioactive TGFβ and no significant difference in total TGFβ. The reason for the difference between these studies could be that the bioassay detected $TGF\beta_1$ and $TGF\beta_2$ activity, as well as other interfering substances (tumour necrosis factor α and β) that may be present in amniotic fluid. The immunoassay used in the current study was specific for $TGF\beta_1$ with less than 2% cross reactivity with other TGFβ isoforms (Promega assay data sheet).

A few studies have been carried out investigating $TGF\beta_1$ levels in abnormalities of pregnancy other than DS. Significantly elevated maternal plasma levels of $TGF\beta_1$ have been associated with pregnancies complicated by pre-eclampsia (Djurovic et al. 1997), and also in pregnant women suffering miscarriage and in non-pregnant women with a history of recurrent miscarriage (Ogasawara et al. 2000). The current study could not find any significant difference between total serum levels of this growth factor between either control and DS groups or pregnant and non-pregnant groups.

It is postulated that altered placental levels of $TGF\beta_1$ may contribute to the altered levels of hCG expression in DS pregnancies. $TGF\beta_1$ has an inhibitory effect on hCG production and cytotrophoblast syncytialisation, a process that is related to the rate of

hCG production (Hay, 1988). It is possible that lower levels of TGFβ_1 could affect this process by allowing more syncytialisation to take place, thereby contributing to increased placental hCG production leading to elevated maternal serum levels of this hormone in DS pregnancies. Increased syncytialisation could also provide a mechanism for increased production and secretion of other placental markers into the maternal serum in DS pregnancies (e.g. inhibin-A). However, not all placental markers show elevated serum levels in DS pregnancies (e.g. PAPP-A, SP-1 and the fetal/placental product UE_3 are reduced) and the magnitude of the changes in variable between different markers is gestation dependant, suggesting a more complex mechanism may be operating.

4.2.3 Conclusions: Growth Factor Study

Following the same procedure as that carried out with the inhibin and activin data, the relationship between EGF and TGFβ_1 levels and previously measured hCG levels was investigated using Pearson's pairwise correlation. There was no significant association between placental and AF levels of TGFβ_1 or placental TGFβ_1 and fβ or ihCG. The levels of neither growth factor were correlated with either fβ or ihCG levels in amniotic fluid. If either EGF or TGFβ_1 were directly related to the increased placental production of hCG, a degree of correlation would be expected between the levels of these factors as was observed for inhibin-A or activin-A and hCG. The fact that no correlation was observed suggests that these factors are unlikely to have a direct effect on the production of hCG although an indirect effect (e.g. modulation of cytotrophoblast differentiation) cannot be excluded.

Although based on a small series of control and DS cases, the majority of trisomic pregnancies selected for study had shown increased hCG levels in routine second trimester screening and might have been expected to show changes in EGF or TGFβ_1 levels if these growth factors were implicated in the alteration of hCG levels. It could be the case, however, that abnormal fetal or placental function is the reason behind the changes in growth factor production, rather than altered growth factor levels being the cause of fetal/placental malfunction in DS. Altered TGFβ_1 and EGF levels could affect the development of either the placenta or fetus in DS pregnancies, however the specific reason for these altered growth factor levels in these pregnancies is unclear as the gene for neither is located on chromosome 21.

4.3 mRNA Quantification

To study hCG production by cultured trophoblast cells, both secretion of the protein and mRNA production had to be measured. These cells are difficult to isolate and culture, consequently RNA samples from these cells were scarce. It was therefore desirable to find an efficient method of mRNA quantification suitable for determining both hCG and a control gene mRNA production by trophoblast cultures. Until recent years, the principal method of specific mRNA quantification was northern blot analysis. This relies on accurate determination of band densities on autoradiographs using an imaging densitometer to compare the quantity of mRNA loaded in each lane. Northern blot analysis is also extremely time consuming if many samples need to be analysed because each RNA sample has to be separated by gel electrophoresis. The sensitivity of this technique is limited and it was found in the current study that only large differences in density could be accurately measured. The main drawback of this technique is the quantity of total RNA required to quantify the specific mRNA of interest in each sample. For one gel 0.5-20μg total RNA is generally required for each sample. In the case of the hCG probe used in this study, a minimum of 5μg was required to produce a detectable signal.

4.3.1 ELISA Based mRNA Assay

This type of assay was commercially available to quantify GAPDH mRNA, which was used in this study as the control gene, but there was not a commercially available kit to quantify hCG mRNA. This meant that the commercially available base kit had to be adapted to allow it to be used to quantify hCGβ mRNA. This method proved to be a quick and easy way to determine the quantity of both mRNA transcripts in each sample. Each assay allowed 40 samples to be analysed in duplicate and only 2μg or 4μg total RNA was required for each well. This meant that duplicate results for both genes of interest could be obtained using a total of 8μg or 16μg total RNA. In contrast a minimum of 20μg total RNA would be required to analyse the expression of both of these genes in duplicate by northern hybridisation analysis. The speed of the assay and the number of samples that could be dealt with at one time meant that over 60 samples could be assayed for both hCG and GAPDH mRNA expression in the time it would take to carry out two northern blots. ELISA based mRNA results were compared to those

obtained using real time quantitative PCR. There was a significant correlation observed between the results obtained using each method, despite the control mRNA differing between methods. This proves that the ELISA based method provides accurate results. The fact that no specialist equipment is required for this method, only a plate reader capable of measuring light absorbance like those used for a standard ELISA, makes this a relatively inexpensive method to use. This is of particular value if there would be no long term continued requirement for measuring levels of mRNA expression.

The main drawback of this method was the time taken to optimise the assay for use with the customised probes. If two commercially available targets were of interest this would not be a problem making this a more attractive method to use. The numbers reported by the custom assay were only relative to a standard set of dilutions of a stock RNA sample. This meant that results were in arbitrary units rather than in atomoles as is reported by the commercially available assays. Despite requiring less RNA than a northern blot, the quantity of RNA required for this method is considerably more than PCR based methods. The quantity of RNA is particularly crucial when tissues or cells to be analysed are scarce. The sensitivity of the custom assay could be further improved by double labelling the DIG labelled probe. This probe only received a 5' DIG label but it would be possible to label both the 5' and the 3' ends of the probe with DIG thereby doubling the number of anti-DIG antibody binding sites and theoretically doubling the sensitivity of the assay. This would decrease the quantity of RNA required to generate a signal but would of course increase the cost of probe synthesis.

4.3.2 Real Time Quantitative PCR

The PCR based method was by far the most sensitive way of determining the level of mRNA expression in samples. Multiplex reactions were carried out so that both hCG and the control gene (PPIA) expression could be analysed concomitantly. Reverse transcription of 2μg RNA provided sufficient cDNA to allow up to eight multiplex reactions, each carried out in duplicate. This is also an extremely quick method. Each two hour PCR reaction can analyse 45 RNA samples. Real time quantitative PCR is the most accurate PCR based method of mRNA quantification because the rate of the PCR reaction is monitored throughout ensuring that all measurements are taken during the logarithmic phase of the reaction. This can often be difficult to achieve when using an

end point method of quantitative PCR analysis particularly if there is large variation in the levels of expression between samples.

The main drawback is the fact that smaller academic departments are unlikely to have access to the TaqMan® which carries out the PCR reaction. The reagents required for each reaction are comparable in price to an ELISA assay meaning that, if the equipment is accessible within a department, this is an economical way of analysing mRNA expression.

4.3.3 Conclusions: mRNA Quantification Methods

The major points to consider when deciding between the three studied methods of mRNA quantification are summarised in table 4.3.3. Probably the most efficient method of determining the level of mRNA expression when the quantity of RNA is limited and a large number of samples have to be assayed is Real Time PCR. Access to the TaqMan® could be difficult because it is expensive to purchase and only departments that regularly quantify mRNA are likely to invest in this piece of equipment. There are other PCR methods using standard PCR machines that can be used. These usually involve stopping the PCR reaction in the logarithmic phase and employing a method of end point quantification. These methods, like Real Time PCR, require careful optimisation and are not quite as reliable as the Real Time method. If a TaqMan® is not available this could provide a viable alternative.

The ELISA method provided a good, reliable alternative to PCR methods of quantification and is certainly quicker, easier and more sensitive than northern blot analysis. Modification of the standard assay was time consuming but following this it was easy and quick to carry out. The base kit of the assay has recently been modified to allow the use of collected cells rather than isolated RNA in the assay. Cells are collected into a lysis solution provided with the assay kit and are added directly to the ELISA plate. This removes the need for RNA isolation procedures, further speeding up the process of assaying mRNA levels in cultured cells. As more targets have pre-made kits available, this will become an attractive method of mRNA quantification particularly in laboratories where large investments for new equipment are not available.

	Northern Blot	ELISA	Real Time PCR
Quantity of RNA	20-40μg	8-16μg	2μg
Minimum Time	3 days	4h	2h
No. of samples assayed at once	10	40	45
Obvious Drawbacks	• Uses radioactivity and other hazardous chemicals. • Limited sensitivity. • Exposure time may need to be increased to several days.	• Limited number of commercially available targets & optimisation of custom target is a lengthy process. • Assay kits are moderately expensive.	• TaqMan® is an extremely expensive investment. • Reagents are moderately expensive.

Table 4.3.3 *Summary of points to consider when comparing three methods of mRNA quantification.*

4.4 Trophoblast Culture

As mentioned in previous sections, the primary sites of synthesis of placental derived maternal serum markers of DS pregnancies are trophoblasts. It was one of the main aims of this project to optimise a method of isolating and culturing cytotrophoblast cells to allow the study of hCG production and secretion by these cells as they differentiate to form syncytium. The method that was chosen was the widely used Kliman (1986) method. Term placentae were used to optimise this method with the ultimate aim of transferring this method to mid trimester DS and control placentae.

4.4.1 Viability and Purity of Trophoblast Cells

Following initial isolation, approximately 1.5-3.5x10^6 cells per gram (wet weight) of tissue were isolated, which were approximately 90-95% pure and greater than 90% viable. This is in line with the previously reported yields and purity by those routinely working with isolated trophoblast cells (Kliman et al. 1986; Bax et al. 1989; Truman et al. 1989; Frank et al. 2000; Frank et al. 2001). Following 24h in culture, the percentage of cells which failed to adhere to the culture surface was typically less than 10% which again is similar to the viability of trophoblast cells previously reported. For many experimental procedures this degree of purity is sufficient and cells without any further purification steps were used in this study to assess the effects of cryopreservation on trophoblast function. It was discovered that

occasional batches of trypsin provided poor cell yields as has been noted before (Frank et al. 2000; Frank et al. 2001).

One problem that was encountered during isolation procedures was clumping of trophoblast cells making them difficult to resuspend and seed onto the culture surface evenly. This problem has previously been reported when using the Kliman method of cell isolation followed by the use of Percoll gradients to further purify cytotrophoblast cells (Karl et al. 1992). It was suggested in the latter study that a further short trypsin/DNase treatment of the cells was an effective solution, however in the current study it was discovered that refrigerating the cells between isolation steps and using cold culture medium until the point when the cells were seeded onto the culture surface eliminated clumping. Another modification, which was made to the original Kliman (1986) method, was to reduce the number of 30min digestion steps from three to two. It was observed that the third digestion step seemed to remove larger clumps of cells from the villous fragments rather than the single cells that were largely removed during the first two digestion steps. These clumps of cells interfered with the Percoll purification step by clumping near the top of the gradient and trapping some of the mononuclear cells thereby preventing their collection. Using two digestion steps resulted in a purer cytotrophoblast isolation without any noticeable reduction in cell yield.

The primary contaminants in the isolated cells were blood leukocytes as indicated by positive reactivity with anti CD45 antibodies. Similar contamination has been reported by others (Blaschitz *et al.* 2000). Further immunopurification of trophoblast preparations resulted in a preparation that was greater than 98% pure. One study has reported that anti HLA immunopurification of trophoblast cells markedly increases their ability to differentiate when compared to non-purified cells as monitored by immunocytochemical staining and hCG and hPL secretion (Cervar et al. 1999). It has been reported that anti CD9 purified trophoblast preparations fail to differentiate properly in culture as monitored by immunocytochemistry and lower hCG secretion, and that these cells require administration of EGF to induce syncytialisation (Morrish et al. 1997). As can been seen from figures 3.2.2.2c and 3.2.2.2d there appeared to be little difference in syncytialisation between pre and post immunopurification cultures in this study. There also appeared to be little difference in the rate of hCG and PLAP production, although there was a tendency towards higher secretion of hCG in immunopurified cells. It is difficult to find a reason for such a variation in results

between these studies. The present study and the study by (Cervar et al. 1999) both used anti HLA class I immunopurification and were based on results from 3 and 17 placentae respectively. The study by Morrish *et al* (1997) used anti CD9 immunopurification and appears to be based on fewer placentae than the (Cervar et al. 1999) study, perhaps a maximum of five placentae for some aspects, although other results appear to be based on only 1 or 2 placentae. Although different antibodies were used to remove contaminating cells in these studies, it is unlikely that this would make any difference to the function of the remaining cytotrophoblast cells. In the current study, it was observed that anti CD9 antibodies did not appear to bind to all contaminating cells. Nevertheless, Morrish *et al* (1997) reported a degree of purity similar to the Cervar (1997) and the current studies, indicating that the different antibodies are unlikely to provide a reason for the altered cytotrophoblast behaviour.

Immunopurified cells were required for studying the effects of interferon on hCG secretion from trophoblasts because it has been reported that leukocytes and macrophages, which are the main contaminants of trophoblast cultures (Blaschitz et al. 2000), produce interferons (De Maeyer and De Maeyer-Guignard, 1998). This would give a background level of interferon, which may interfere with the study of the effects of administered interferon on these cells. The use of macrophage conditioned medium has also been shown to affect hCG secretion from trophoblast cultures although one study showed an increase in hCG secretion (Khan et al. 2000) while another showed a decrease in hCG secretion (Cervar et al. 1999) when macrophage conditioned medium was used to culture cells.

4.4.2 Effect of Cryopreservation on Cells

Due to the uncertainty inherent in the time of collection of placentae for cytotrophoblast cell isolation and the difficulties of scheduling a series of 24h medium changes, it would be desirable if isolated cells could be cryopreserved for recovery at a convenient time. This would especially be the case where placentae from mid trimester TOP were being collected as these often occur out of normal working hours. The effect of cryopreservation on isolated term placental cytotrophoblast cells was therefore investigated to assess whether cytotrophoblast cells could be stored in this way to allow recovery at a convenient time point, which would allow a set of experiments to be carried out simultaneously on different cultures.

It is clear from section 3.3.1.2 that trophoblast cells do not behave normally after recovery from cryopreservation. Lower rates of hCG secretion were noted in cryopreserved cultures as was failure of morphological differentiation assessed by light microscopy, although no difference in PLAP secretion was observed. There was also no difference in the quantity of DNA or protein in the culture wells between fresh and cryopreserved cells. This indicated that similar numbers of cells remained in the wells during the culture period of both fresh and frozen cells. The difference in hCG secretion cannot therefore be attributed to some effect caused by correcting the hCG results by total protein measurements but must be due to alterations in the secretion of this hormone. One possibility that could account for this difference could be that contaminating cells survive the cryopreservation procedure better than cytotrophoblast cells and begin to increase in number during the culture period. ICC analysis of vimentin positive cells at 24h and 96h after recovery of cells from frozen storage did not reveal any obvious increase in contaminating cell number when compared with the matching fresh culture.

The freezing protocol used was that described by Yui *et al* (1994) who did not report such problems. Other groups have also reported results on trophoblast cells recovered from frozen storage, however no definitive study on the effects of cryopreservation on trophoblast function has been reported. The reason why trophoblast cells perform so poorly following cryopreservation could be because these are primary cells that do not grow and divide in culture, only differentiate. Some cells would be expected to die following cryopreservation. If cells actively divide, then cells lost during this process will be replaced but this would obviously not be the case where trophoblasts are concerned. Trophoblast cells also have a limited lifespan, consequently it is impossible to give the cells some time to recover in culture before working with them as could be done with actively dividing cells. It was decided that for the purpose of this study, only freshly isolated cells would be used because cryopreserved cells did not behave in a predictable manner following recovery. Perhaps for some studies cryopreserved cells would be suitable but for studying the effects of factors on hCG secretion, using a system where the hCG secretion is obviously different from that seen in freshly isolated cells does not seem appropriate.

4.5 Increased hCG in DS Pregnancies

4.5.1 Syncytialisation of DS Cultured Cytotrophoblast Cells

Since the commencement of this research, further studies have been reported where the mechanisms behind altered maternal serum levels of hCG in DS pregnancies have been examined. One group have studied cultured trophoblast cells isolated from control and DS placentae. The formation of syncytium, as the culture time progressed, was monitored by immunocytochemical analysis of desmoplakin localisation. Desmoplakin surrounds individual cytotrophoblasts but reactivity diminishes as the cytotrophoblast cells fuse to become a continual syncytial layer (see fig 3.2.2.2c for the normal situation). It was found in both reports by this group that DS cells failed to syncytialise as control trophoblast cells did. This failure of syncytialisation in DS cultures was accompanied by lower rates of hCG mRNA production and protein secretion (Frendo et al. 2000; Evain-Brion et al. 2000). These results were based in the first report on 8 control and 8 DS placentae collected from second trimester terminations (Evain-Brion et al. 2000). The second report was based on 10 control and 15 DS placentae collected from both the second and third trimesters (Frendo et al. 2000). These results on cultured cells were confirmed in both reports by measuring the mRNA expression of markers, which increase during the process of syncytialisation, in total placental homogenates from the 8 control and DS placentae using real time PCR analysis. They found that levels of hPL, hCGα, hCGβ, placental growth hormone and leptin mRNA were all significantly lower in DS placentae (Frendo et al. 2000; Evain-Brion et al. 2000) suggesting that *in vivo* formation of syncytiotrophoblast is also defective. They conclude that these findings may aid in understanding the reasons behind elevated maternal serum levels of hCG in DS pregnancies. This was a particularly surprising finding since most studies carried out prior to these studies indicated that elevated maternal serum levels of hCG largely reflected increased placental production of the hormone (Eldar-Geva et al. 1995; Newby et al. 1997). This discrepancy with the previous studies on placental production of hCG were not addressed by Frendo *et al* (2000) with the study of Eldar-Geva *et al* (1995) only being cited to support the finding of reduced syncytialisation in DS pregnancies.

The larger study carried out by this group includes some third trimester placentae which would probably not be expected to show altered levels of hCG because these alterations are gestation specific. The second trimester maternal serum screening programme based on maternal serum hCG levels is most reliable at 15 weeks of gestation when the largest difference in hCG levels between control and DS samples is evident. The difference between control and DS serum levels of hCG decline as gestation progresses until around 20-22 weeks of gestation when little difference between control and DS groups is observed. Consideration must therefore be given to the gestation at which placentae are obtained when investigating the alterations in maternal serum levels of placental derived markers.

The placentae selected as controls for the above studies (Frendo et al. 2000; Evain-Brion et al. 2000) were not from pregnancies terminated for psycho/social reasons, which would perhaps proved the most appropriate normal controls, but were obtained from pregnancies with abnormalities other than chromosomal, including severe bilateral or low obstructive uropathy, skeletal abnormalities and cardiac anomaly. Although these abnormalities are fetal and not placental they could conceivably alter the secretion of hCG when compared to the normal situation. No information is given as to the maternal serum marker levels of these pregnancies. If this information was reported, as well as confirming that the "control" placentae were from appropriate pregnancies, it would be helpful in the interpretation of the placental results because the reported differences in placental marker expression could be correlated with the situation in maternal serum.

The same group suggested that this failure to syncytialise was due to over-expression of the chromosome 21 product, copper zinc superoxide dismutase (SOD-1) (Evain-Brion et al. 2000; Frendo et al. 2001). Both reports indicated that DS placentae expressed higher levels of SOD-1 than gestation matched controls. They also transfected normal term trophoblast cells with a plasmid containing the SOD-1 gene that caused increased SOD-1 expression similar to that in DS cells. It was observed that the transfected cells showed inhibition in syncytialisation and hCG secretion similar to that found in DS trophoblasts. They also found that levels of hPL, hCGα, hCGβ and placental growth hormone but not leptin mRNA were significantly lower in the SOD-1 transfected cells. Because transfection of cells with SOD-1 expressing vector was not totally effective, the second study tagged SOD-1 with green fluorescent protein (GFP) so the transfected

protein could be visualised in trophoblast cells in culture. They noted that the cells that showed positive GFP-SOD-1 reactivity did not syncytialise while cells on the same culture plate that did not express the GFP-SOD-1 fused to form syncytium. This study indicates that the defects in syncytialisation observed in DS placental cells could be attributed in part to the over expression of SOD-1 (Evain-Brion et al. 2000; Frendo et al. 2001). It is unclear however, how many placentae these results are based upon. It is thought that over-expression of SOD-1 leads to increased oxidative stress. This could then cause damage to the trophoblast cells preventing syncytialisation, and *in vivo* may cause increased proliferation of trophoblasts explaining the observation of increased numbers of cytotrophoblast cells in DS placental sections as has been noted previously (Roberts et al. 2000).

The results of these studies (Frendo et al. 2000; Evain-Brion et al. 2000) are surprising since they conflict with the findings of previous studies on hCG levels in DS placentae which show elevated placental protein levels and mRNA expression of hCG in DS pregnancies (Eldar-Geva et al. 1995; Newby et al. 1997). As mentioned earlier these differences between studies were not addressed in the recent studies (Frendo et al. 2000; Evain-Brion et al. 2000) although the reason why maternal serum levels of hCG showed an increase in hCG while the placenta showed decreased levels in DS were discussed. It was suggested by Frendo *et al* (2000) that the increased level of hCG observed in maternal serum was due to increased glycosylation of hCG making it less susceptible to degradation rather than increased production of the hormone by placental cells.

4.5.2 Hyperglycosylated hCG (H-hCG)

H-hCG has the same peptide structure as ihCG but has larger N- and O- linked oligosaccharides on the β subunit. H-hCG is also known as invasive trophoblast antigen, alluding to the possible source of H-hCG. It is thought that invasive cytotrophoblast cells secrete H-hCG rather than villous trophoblasts, as is the case for ihCG (Cole et al. 1999). This cell specific production also explains the gestational variation in the contribution of H-hCG to the total hCG secreted during pregnancy, with peak production at the point in gestation when peak cytotrophoblast invasion is occurring (table 4.5.2).

Gestation	ihCG	β-Core hCG	Contribution of H-hCG
	(nmol/mmol creatinine)		
4-6 weeks	7-8	9-11	26%
6-8 weeks	8-9	11-15	11%
8-14 weeks	9-2	15-40	2.9%
14 weeks-term	2-3	15-5	2%

Table 4.5.2 *ihCG and β-core hCG concentrations in maternal urine throughout normal pregnancy and the percentage contribution of H-hCG. Values obtained from de Medeiros et al. (1992) and those cited as unpublished data by Shahabi and Cole (Cole et al. 1999).*

H-hCG has been reported as a useful maternal urine screening marker for DS pregnancies with a greater degree of elevation in urine (up to 9MoM) when compared with other forms of hCG such as fβ or β core hCG (Cole et al. 1998; Cole et al. 1999; Cuckle et al. 1999b; Bahdo-Singh et al. 2000). These studies of H-hCG mainly focused on the second trimester of pregnancy, although a smaller elevation in first trimester urinary H-hCG to 3.6MoM has been reported (Weinans et al. 2000). In the second trimester, only 2 to 2.9% of control hCG production is accounted for by H-hCG (table 4.5.2). The placental studies (Evain-Brion et al. 2000) that suggested overglycosylation rather than overproduction of hCG was the main cause of elevated maternal serum levels of this hormone also focused on the period in gestation where only 2% of total hCG is hyperglycosylated. It is fair to speculate that in the DS cases used by Evain-Brion et al. (2000) perhaps up to nine times more H-hCG was being produced by cytotrophoblast cells based on the reported degree of elevation in maternal urine of DS pregnancies. The results of the placental studies (Evain-Brion et al. 2000) suggest a 6-20 fold reduction in hCG production by DS placentae. Although it is possible that the increased levels in hCG are in part due to altered degradation of H-hCG, it is difficult to attribute increased hCG levels entirely to this mechanism particularly if placental hCG secretion is reduced as was suggested (Evain-Brion et al. 2000). If slower degradation was the only reason behind elevated maternal serum levels of hCG rather than over production by the placenta, then decreased levels of hCG would be expected in maternal urine samples from DS pregnancies at some point in gestation, although the levels could begin to increase as more hCG is eventually broken down. This is not the case. Elevated maternal urine levels of both fβ-hCG and β core hCG (the

terminally degraded form of hCG) have been widely reported in both the first and second trimesters of DS pregnancies. This indicates that synthesis of this hormone is more likely to be increased rather than inhibition of H-hCG degradation being the reason behind elevated maternal serum levels of hCG.

Further studies by the same group (Guibourdenche et al. 2001) reported that hCG secreted from cultured DS trophoblasts had a significantly greater fraction of H-hCG than controls. The mRNA levels of two enzymes involved in the glycosylation of proteins (sialyl-transferase-1 and fucosyl-transferase-1) were also studied in control and DS trophoblast cultures. Levels of mRNA for both enzymes were significantly elevated in DS cells suggesting altered glycosylation of hCG by DS villous trophoblasts. It is thought that H-hCG is primarily produced by invading cytotrophoblast cells rather than villous trophoblasts (Cole et al. 1999). The study of Guibourdenche et al. (2001) that reported altered production of H-hCG in DS examined cells isolated from placentae using a protocol that results in preparations of mainly villous cytotrophoblast. As has been reported, these isolations of DS cytotrophoblast cells do not fuse to form syncytium over the culture period as control cultures do and produce a marker of invasive cytotrophoblast (H-hCG). It could be that different populations of cells are being isolated from the control and DS placentae or that once isolated, DS cytotrophoblast cells follow the invasive pathway rather than the pathway of differentiation as the control cultures did. These studies do not further immunopurify their cytotrophoblast preparations, and only vimentin and cytokeratin 7 immunocytochemistry is used to determine the purity of the isolated cells. EVT express cytokeratin 7 and the limited antibody panel used in these studies would not be able to determine the specific lineage of the cytotrophoblast cells. It is therefore possible that for some reason the DS cytotrophoblast preferentially underwent the invasive route while the control cytotrophoblast syncytialised. This would not only explain increased H-hCG production but also the observation of decreased hCG and hPL secretion (markers of syncytialisation) by the DS cytotrophoblast cultures. It has been suggested that placental immaturity could be a reason behind increased hCG production because levels in DS maternal serum are similar to those in control pregnancies two to three weeks earlier in gestation (Canick et al. 1988; Chard, 1991; Waller et al. 1993). If this was the case then the number of cytotrophoblast cells destined for the invasive phenotype may be greater in DS pregnancies because invasion decreases as pregnancy advances.

4.5.3 LH/hCG Receptor Expression in DS Placentae

A further study (Jauniaux et al. 2000), which supports previous findings on increased β-hCG subunit expression in DS placentae, also reported increased LH/hCG receptor expression in DS placentae from 12-16 weeks gestation. Receptor expression was analysed by immunohistochemistry and *in situ* hybridisation. LH/hCG receptors and receptor mRNA were primarily localised to the syncytiotrophoblast layer with weaker staining of the cytotrophoblast cells. Increased hCG expression has previously been shown to have a negative regulatory effect on the synthesis of LH/hCG receptors. However, in the first trimester of pregnancy, LH/hCG receptors are truncated and probably do not function as the full length receptor would. It would be expected that in DS pregnancies, which have a high hCG concentration, that the elevated hCG would down regulate receptor expression leading to decreased receptor expression in DS placentae. Because the opposite was observed in DS placentae by Jauniaux et al. (2000) it was suggested in this as well as previous studies that increased hCG levels and increased LH/hCG receptor expression was merely a reflection of placental immaturity in DS pregnancies. If the maturity of the DS placenta was equivalent to a normal placenta of two to three weeks earlier in gestation then the self regulatory mechanism of hCG on its receptors may not be functional yet in the DS placentae.

4.5.4 Placental/Fetal Immaturity in DS

Placental and/or fetal immaturity has previously been suggested as a reason behind altered maternal serum marker levels (Canick et al. 1988; Chard, 1991; Waller et al. 1993). Estriol precursors and AFP are synthesised by the fetus and it has been suggested that immaturity of fetal organs, in particular the adrenal cortex and liver could be the reason behind reduced maternal serum levels of both of these markers in DS (Canick et al. 1988). It was observed that the reduction in maternal serum AFP is consistent with the concentration observed around two to three weeks earlier in gestation (Canick et al. 1988; Waller et al. 1993). One study analysed the association between maternal serum AFP and fetal birth weight and found no association between restricted birth weights and reduced maternal serum AFP (Waller et al. 1993). A similar observation was made regarding the elevation in maternal serum hCG, with the degree of elevation matching the concentration observed earlier in pregnancy. Because hCG is a placental product, placental rather than fetal immaturity is the suggested cause for elevated maternal serum

levels of this marker (Wald et al. 1988). Placental immaturity could also contribute to reduced maternal serum UE_3 levels and could explain increased levels of inhibin-A.

However, other placental derived maternal serum markers of DS do not follow this pattern. The maternal serum concentration of SP-1 in normal pregnancy increases steadily throughout pregnancy, but second trimester maternal serum SP-1 in DS is significantly increased (Graham et al. 1992). If placental immaturity was the sole reason behind altered marker levels then SP-1 would be expected to be decreased in DS. There is little pathological evidence of placental or fetal immaturity in DS. Normal fetal and placental size and weight has been reported previously throughout gestation (MacAfee et al. 1970; Kučera, 1971; Wald et al. 1993; Kuhn et al. 1995). At the microscopic level, irregular maturation of trisomic placentae has been reported including immature villi (table 1.4.4.2). Taken together, it appears that fetal/placental immaturity is unlikely to be the main reason for altered maternal serum marker levels in DS pregnancies although defects in the maturation of specific placental cells (e.g. cytotrophoblast) could have a role to play.

Although there have been reports to the contrary, most of the available evidence supports increased placental production of hCG as the reason behind elevated maternal serum levels of this marker in DS pregnancies.

4.5.5 IFN and hCG Secretion from Trophoblast Cultures

IFNα and IFNβ are the predominant forms of IFN found in the placenta and fetus during pregnancy (Chard et al. 1986; Bennett et al. 1999). It is thought that these forms of IFN play a role in protecting the fetal/placental unit from rejection by the mother. The placental membranes have a high concentration of IFN type I receptors and they are specifically localised to the syncytiotrophoblast as is IFNα, indicating that IFNα and IFNβ have an important role to play at the fetal/maternal interface (Howatson et al. 1988; Paulesu et al. 1991; Paulesu et al. 1997; Bennett et al. 1999). If prevention of immune rejection is the main function of IFNα, the syncytiotrophoblast is an obvious target since this forms the main barrier between mother and fetus. In contrast, IFNγ levels are low during pregnancy (Vassiliadis et al. 1998) although first trimester trophoblasts have been shown to express IFNγ and IFNGR1 (Paulesu et al. 1994). Maternal serum concentrations in early pregnancy are similar to those in the non-

pregnant state and rise only slightly as gestation progresses to a peak during labour. Elevated maternal serum IFNγ concentrations are associated with first trimester spontaneous abortion (Vassiliadis et al. 1998).

Increased IFN-R1 or IFNGR2 expression in DS placentae could have some bearing on the early rejection of some of these fetuses. The increased receptor expression could also have an effect on the production of maternal serum markers of DS pregnancies, particularly those synthesised by placental trophoblastic cells (e.g. hCG, inhibin-A and PAPP-A). There have been no previous reports of the effects of IFNα or IFNβ on the synthesis of such markers by placental trophoblast cells although IFNγ has been reported to reduce hCG secretion from term trophoblast cultures (Marth et al. 1989). There has been one report on the effects of IFNα administration to hCG secreting bladder carcinoma cell lines (Iles and Chard, 1989). This study found that IFNα induced hCG secretion from these cells in a dose dependant manner. It was postulated that this effect may also be present when IFNα was administered to cytotrophoblast cell cultures and it was the aim of the current study to test this hypothesis. If IFNα did, in fact induce this response then receptor over-expression in DS placental trophoblasts could perhaps provide a reason for increased hCG secretion from DS placentae.

Section 3.4 suggests that despite possessing functional IFN receptors, placental trophoblasts are insensitive to IFNα administration at term as a mechanism of altered hCG secretion. IFNα plays an immunomodulatory role in many cell types, inducing the expression of MHC class I antigens. However, IFNα fails to induce this response when administered to term trophoblast cell lines (JEG-3 and JAR) (Cross et al. 1999) and it was discovered that this insensitivity to IFNα was due to the failure of a step in the IFNα signalling pathway. Upon IFNα stimulation, receptors were activated and STAT signalling was induced, however the formation of the ISGF3/p48 complex which should enter the nucleus and bind the ISRE failed. Whether this response is missing in normal human trophoblasts and whether hCG release from trophoblasts would be involved in this particular pathway, is unknown. It is apparent from the current study that term placental trophoblasts do not respond to IFNα administration by secreting more hCG, however cells from the mid-trimester may behave in a different way. It is at this stage of pregnancy when differences in hCG levels between DS and controls are largest and therefore the peak responsiveness to the factor that causes this increase would be

expected. The study of trophoblasts isolated from mid-trimester placentae is required before the postulated effect of increased IFN receptor expression in DS placentae on hCG production could be discounted.

4.6 Future Research

4.6.1 Placental Production of DS Maternal Serum Markers

The results of this and previous studies on placental function clearly indicate that the production of cytokines, growth factors and hormones is abnormal in placentae from DS pregnancies. The current study indicated that in the case of inhibin-A and activin-A the alterations in maternal serum levels of these markers largely reflect increased production by the placenta. These findings match the earlier reports studying maternal serum levels of hCG in relation to placental production of this hormone (Eldar-Geva et al. 1995; Newby et al. 1997). There have, however, been several studies relating to placental production of hCG in DS pregnancies that are not in accordance with these findings (all studies are summarised in table 4.6.1). These differences in findings indicate that further work in this field is required to elucidate whether changes in maternal serum marker levels in DS pregnancies reflect altered placental production or if a more complex mechanism exists.

	Eldar-Geva	Brizot	Newby	Jauniaux	E-B/Frendo
Plac ihCG			Sign. elevated	Small increase	
Plac fβhCG			Sign. elevated	Sign. elevated	
Plac αhCG				No difference	
MS fβhCG		Sign. elevated	Sign. elevated		
MS ihCG			Sign. elevated		
hCG IHC			Stronger		
hCG secretion *in vitro*	Sign. elevated				Sign. decreased
β mRNA	Large increase	No difference			Sign. decreased
α mRNA	Small increase	No difference			Sign. decreased
Cytotrophoblast differentiation	Inhibited				Inhibited/ Delayed
LH/hCG receptor				Stronger	
No. of Cases	4 DS, 3 C	11 DS, 30 C	51 DS, 52 C	8 DS, 42 C	15 DS, 10 C
Gestations	17-23 weeks	11-15 weeks	10-24 weeks	12-16 weeks	12-35 weeks

Table 4.6.1 *Summary of findings from studies of placental hCG production in DS pregnancies. Papers summarised are: Eldar-Geva et al (1995); Brizot et al (1995); Newby et al (1997); Jauniaux et al (2000); Evain-Brion et al (2000); Frendo et al (2000). Plac=placental, MS=maternal serum, sign=statistically significant and C=control.*

4.6.2 Chromosome 21 Specific Markers and the DS Placenta

The current study used the analysis of growth factor levels as a method of analysing fetal/placental function in normal and DS pregnancies. Although this method did yield some interesting observations about the variations in growth factor levels in these pregnancies, uncertainty remains as to whether these differences are a cause of altered fetal/placental function in DS or are themselves the effect of altered function due to a different mechanism.

An alternative approach in future studies would be to focus on the effects of altered expression of chromosome 21 specific markers on the function of the placenta in DS

pregnancies. In the latter part of the current study the possible effect of increased expression of chromosome 21 products, IFN-R1 and IFN-R2, on hCG secretion by trophoblast cultures was investigated. Although this study indicated that IFNα administration did not seem to alter hCG secretion from these cells, only term placentae were available for analysis. Further analysis, using mid trimester placentae may yield a different result since, as noted above, this is the stage of pregnancy where the median shift in hCG levels in DS pregnancies is at a maximum. The effect of IFNα administration to DS trophoblasts at this stage would provide further insight into the differences in IFN-R1 and IFN-R2 expression and hCG secretion from these cells. Similar investigations could be carried out using IFNβ to determine its effects on hCG secretion from trophoblasts since it shares the same receptors as IFNα.

Other chromosome 21 specific markers that could be studied in DS placentae include SOD1, IFNGR2 and STCH. SOD1 (as examined by Frendo *et al.* (2000)) is normally protective against cellular oxidative damage caused by free radicals. At around 10-12 weeks of gestation, there is a dramatic increase in oxidative stress in the placenta due to the massive increase in oxygen tension caused by the onset of maternal circulation into the intervillous space. It is thought that SOD1 has an important role to play in minimising damage to the fetus caused by free radicals during this time and throughout the rest of gestation. Over-expression of SOD1 could prove protective to the placenta and fetus during this period of pregnancy. However, increased SOD1 activity results in increased production of hydrogen peroxide, and if this accumulates in cells due to inadequate clearance by glutathione peroxidase or catalase, this could, in turn, lead to increased cellular damage rather than extra protection caused by SOD1 over-expression. Increased damage to trophoblast cells caused by over-expression of SOD1 was the reason proposed by Frendo *et al* (2000; 2001) for reduced syncytialisation and reduced hCG secretion from DS trophoblast cultures. To determine if the situation *in vivo* matches the *in vitro* results reported by Frendo et al. (2000; 2001), investigation of the localisation of SOD1 in control and DS placental sections and the concentration of SOD1 and its products in DS and control placentae could be areas area of future research.

The IFNGR2 is required for cells to respond to IFNγ. The effects of IFNγ on hCG secretion has been examined in at least two studies (Marth et al. 1989; Yanushpolsky et al. 1993). It was noted that IFNγ administration caused a reduction in hCG secretion

from normal term trophoblasts but no alteration in hCG secretion from JAR cells. Neither this factor nor its receptors have been studied in DS placentae and both may provide an interesting target for future study.

Despite being outside the DS "critical region" of chromosome 21 it is proposed that STCH may be an interesting factor to study in DS placentae since the majority of DS cases would probably have an extra copy of this gene. STCH plays an important role in the normal protein folding events within cells (see section 1.6.4). Since most factors that are elevated in the maternal serum of DS pregnancies are proteins, STCH over expression may have a role to play in this elevation in protein marker production. If a difference was found in the function of STCH in DS pregnancies having a trisomy of the majority of chromosome 21, DS pregnancies not having a trisomy of this area of chromosome 21 could then be studied to determine the importance of this factor in the pathophysiology of DS pregnancies.

4.6.3 Conclusion

Without the intervention of screening and prenatal diagnosis, around 800 children with Down's syndrome would be born each year in the UK. As primary prevention is not possible, avoidance of an affected birth depends upon prenatal recognition of Down's syndrome in the fetus and selective termination of affected pregnancies. In the last decade, progress has been made in the non-invasive identification of features of the Down's syndrome pregnancy which mark it out as different from unaffected pregnancies. These include biophysical markers in the first trimester (eg. nuchal translucency) and several biochemical markers in the mother's blood in the first and second trimesters. Currently, routine screening is carried out mainly in the second trimester using combinations of two or three maternal serum markers to identify pregnancies at increased risk. This approach is neither 100% sensitive nor specific and the predictive value of combinations of markers depends upon the interpretation of the magnitude of the change in marker concentration compared to the base-line levels found in unaffected pregnancies. There have been few studies carried out to discover the mechanisms that underlie these marker changes and this has imposed certain limitations on the process of selection of the best markers.

The markers currently in use have been selected largely on an empirical basis, often depending on the availability of suitable assays. Many more, as yet unexplored, pregnancy markers are present in maternal serum and some may prove to be more effective for screening than those currently in use. A more informed selection of new markers might be possible if the effect of the additional gene dosages, due to trisomy of chromosome 21, on the production and secretion of pregnancy markers into the maternal circulation was more fully understood. Recently a few studies have appeared in the literature with the focus of the research on the placenta, the site of production of most of the screening markers in routine use, and the interface between fetus and mother.

Until recently, the function of the normal placenta was primarily studied at the anatomical level rather than at cellular level; consequently the control of hormone and cytokine production in the normal human placenta has been the subject of investigations for only a relatively short period. The recent advances in isolating and culturing placental cytotrophoblast cells have allowed these cells to be monitored *in vitro* as they differentiate to form syncytium. From numerous studies it is well established that trophoblast cells are the main site of production of many placental proteins present in maternal serum (eg. hCG, PAPP-A, inhibin-A, activin-A, SP-1, PLAP, hPL) and that the rate of cytotrophoblast differentiation is closely related to the rate of production of many of these proteins. Since the characteristic changes in DS pregnancies are observed for multiple markers and none has a structural gene on chromosome 21, simple gene dosage effects can be ruled out. Rather, a more generalised effect due to increased dosage of one or more as yet unidentified genes on chromosome 21 on cytotrophoblast maturation seems more likely. Recent studies using trophoblast cultures have uncovered many factors that affect the *in vitro* differentiation of cytotrophoblasts and hCG production at different periods of gestation. These factors include cytokines (eg. interleukins), hormones (eg. insulin and hCG), and growth factors (eg. EGF, TGFα and TGFβ).

Evidence for an effect on trophoblast maturation was sought in the first phase of the present study by investigation of factors that are known to affect hCG secretion. This pointed to changes in the levels of specific placental growth factors and cytokines in DS pregnancies reinforcing the idea that there is disruption of the complex mechanism controlling placental growth and function. As noted above, the basis of such changes

must lie in the additional dosage effect of certain chromosome 21 genes and two candidates, INF-R1 and INF-R2, have been investigated in the present study using term trophoblast cultures. Although no evidence of an enhanced effect on trophoblast differentiation or hCG secretion by administration of IFNα was found, caution must be exercised in the interpretation of such results as the data were obtained at term and there is abundant evidence that the marker changes are gestation related. Such cautions also apply to reports in the literature of other studies (e.g. on SOD1) where gestation effects were not adequately controlled for. It is clear from the present study and others that many variables influence the levels of the markers used to monitor pregnancies for DS and this increases the difficulty of interpretation of results.

The mapping of the full human genome has uncovered a wealth of candidate genes on chromosome 21 although the function of many of these genes is unknown. Perhaps one of these genes could provide the key to answer the DS pathophysiology question and lead to more effective screening in pregnancy.

Section 5

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